Understanding the physiology of combined salinity and waterlogging tolerance in barley

Zhinous Falakboland

University of Tasmania, Australia

Submitted in fulfilment of the requirement for Degree of Master of Science

June 2016

Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any tertiary institution and to the best of my knowledge contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.

Zhinous Falakboland

This thesis may be made available for loan and limited copying in accordance with the Copyright Act 1968.

Zhinous Falakboland

University of Tasmania

Hobart, 30 June 2016

Abstract

The world population is expected to reach over 9.3 billion by 2050 prompting the need to increase agricultural food production by 100%. At the same time, agricultural lands globally are suffering from human induced and natural environmental stresses such as salinity and waterlogging. Soil salinization is affecting more than 800 million hectares (about 6 percent) of the land. While some of this land is naturally saline other parts have become saline as a result of secondary salinization caused by irrigation. According to the FAO, 11% of irrigated lands (about 34 million ha) are suffering from secondary (human-induced) salinity. As the growth of most agricultural crops is strongly reduced by high concentrations of salt in the soil, economic penalties are high, and so is the threat to global food security. About 60-80 million hectares of land are affected to some extent by combined waterlogging and salinity stress. Waterlogging reduces the available air in the soil and has a profound effect on plant growth. These two stresses are often interrelated, as waterlogging can lead to salinization by transporting the salts to the surface. In many parts of the world (e.g. Australia, USA, Pakistan, India, Iran, Thailand and Egypt) these two environmental stresses coexist.

While the physiological and molecular mechanisms of plant responses to each of these environmental constraints have been studied in detail, the mechanisms underlying plant tolerance to their combined stress have not been well understood. This gap in knowledge is jeopardizing the success of breeding programs and has to be bridged. The current study focuses on plant physiological traits under combined stresses compared to separate stresses and no stress.

To address the whole plant physiological mechanisms involved in plant's adaptation to combined waterlogging (WL) and salinity (NaCl) stress, 12 barley varieties contrasting in salinity stress tolerance were grown in potting mixture under controlled light-temperature glasshouse conditions. Two weeks of NaCl, WL and combined WL/NaCl stresses were applied to barley plants. The damage index scoring system was used to evaluate the overall effects of NaCl, WL and combined WL/NaCl on the growth and agronomical characteristics of barley plants, based on the extent of chlorosis and necrosis in the shoot. A 0 to 10 scaling system was used; with grade 0 given to plants that showed no visual symptoms of stress and 10 representing dead plants. Damage symptoms were much stronger in plants under combined WL/NaCl stress compared to separate stresses. The shoot biomass, chlorophyll

content, maximum photochemical efficiency of PSII and shoot K^+ content were significantly reduced under WL/NaCl conditions, while shoot Na^+ content increased. Plants exposed to salinity stress showed less damage indexes compared to WL. Chlorophyll fluorescence Fv/Fm value showed the highest correlation with the plant damage index under WL/NaCl conditions (R = -0.751) compared to other measured physiological traits and was nominated as a good parameter to rank the tolerance of varieties. The average fresh weight was reduced by 73 ± 2, 52 ± 1 and 23 ± 2 percentage of control upon NaCl, WL and combined WL/NaCl treatments, respectively. Chlorophyll content (SPAD values) of the oldest leaf after 10 days of treatments was also used as a proxy for stress tolerance. Based on these findings, barley varieties were divided into two groups. Genotypes having relative SPAD values less than 10% for combined WL/NaCl treatment were classified as sensitive, and those having more than 60% were classified as tolerant. Amongst these, varieties Yerong, ZUG293 and YYXT were found to be the most tolerant.

Na^+ content under control conditions was 97 ± 10 µmol/g, and increased to 1519 ± 123, 179 ± 11 and 2733 ± 248 µmol/g under NaCl, WL and combined WL/NaCl stresses, respectively. K^+ content was 1378 ± 66, 1260 ± 74, 1270 ± 79 and 411 ± 92 µmol/g under control, NaCl, WL and combined WL/NaCl stresses, respectively. Generally, the adverse effect of WL/NaCl stress was much stronger in salt-sensitive varieties such as ZUG403 and Naso Nijo compared to more tolerant varieties such as ZUG293 and YU6472. In general, coexisting waterlogging and salinity provoked a combination of the effects of each stress applied autonomously, even though WL had a greater contribution in limiting factors compared to salinity.

To study and compare the plant shoot and root response to combined WL/NaCl stress, hydroponic experiments were designed. Eight barley varieties contrasting in salinity stress tolerance were grown in half-strength Hoagland solutions for six days. The seedlings were assigned to separate and combined NaCl and WL treatments after 8 (first sampling) and 16 days (second sampling) of stress. Average shoot fresh weight on the first sampling was reduced by 59 ± 5, 55 ± 2 and 41 ± 2 percentage of control upon NaCl, WL and combined WL/NaCl treatments, respectively, while it was only 36 ± 5, 24 ± 2 and 12 ± 1 percentage for the same treatments on the second sampling. The shoot fresh weight changes of the WL/NaCl treated plants correlated more with effect of WL alone and it is suggested that biomass is more limited by hypoxia than salinity in shoots. Average root fresh weight on the first sampling was reduced by 73 ± 6, 46 ± 4 and 30 ± 2 percentage of control upon NaCl, WL and

combined WL/NaCl treatments, respectively, while it was 58 ± 6, 39 ± 3 and 11 ± 1 percentage for the same treatments on the second sampling. Chlorophyll content SPAD value on the second sampling was the lowest under WL/NaCl followed by that in WL-treated plants and then by NaCl-treated plants. Chlorophyll content was used as a proxy for tolerance, therefore varieties YYXT and TX9425 that showed the highest chlorophyll content at both the first and second samplings under combined WL/NaCl conditions were assigned as the most tolerant varieties under WL/NaCl conditions.

Shoot Na^+ content was 95 ± 45, 1736 ± 257, 426 ± 91 and 4401 ± 97 µmol/g under control, NaCl, WL and WL/NaCl treatments, respectively, and shoot K^+ content was 2572 ± 127, 1327 ± 96, 1649 ± 117 and 1321 ± 98 µmol/g under the same treatments. Root Na^+ content was 540 ± 188, 1374 ± 211, 348 ± 39 and 782 ± 210 µmol/g under control, NaCl, WL and WL/NaCl treatments, respectively, and root K^+ content was 2150 ± 328, 397 ± 68, 977 ± 27 and 105 ± 18 µmol/g for the same treatments. In comparing the effects of stresses on root and shoot ionic relations it was concluded that the major limiting factor in root performance was its ability to retain K^+, while shoot performance was more limited by Na^+ increase. Shoot osmolality under WL/NaCl significantly correlated with WL while root osmolality under WL/NaCl highly correlated with NaCl treatment.

To provide some further insights into underlying physiological mechanisms, non-invasive ion-selective microelectrode measurements (the MIFE technique) were used. Selected barley varieties from glasshouse experiments representing low (ZUG403), medium (Gebeina) and high (YU6472) tolerance to combined WL/NaCl stress were used for K^+ and H^+ flux measurements. The tolerant variety YU6472 showed K^+ uptake under all conditions while ZUG403 and Gebeina showed K^+ efflux in response to all three stress conditions, with the biggest efflux detected from ZUG403 roots. These findings strongly suggest that the root's ability to retain K^+ under combined stress conditions is a critical determinant of a plant's adaptive potential to saline flooded soils.

Comparing root ion flux profiles between intact plants and plants with excised coleoptiles has revealed that oxygen transport from the shoot to the root plays an important role in root K^+ retention. Oxygen transport from the shoot to the root provides sufficient oxygen to fuel H^+-ATPase and maintain membrane potentials negative enough to prevent K^+ efflux via depolarization-activated outward-rectifying GORK channels.

It is concluded that WL/NaCl stress is more severe than either salinity or WL stress alone, and the combined effect of WL and NaCl is synergistic but not additive. It is also shown that tolerance to combined WL/NaCl stress is determined mostly by sensitivity to WL. Taken together, combined morphological, physiological and electrophysiological data clearly indicate that plant K^+ ionic relations are more critical than Na^+ in explaining the tolerance to combined WL/NaCl stress in barley.

Acknowledgements

I would like to take this chance to express my deepest appreciation to my primary supervisor, Professor Sergey Shabala, who has the attitude and the substance of a genius and has continuously and convincingly conveyed the spirit of adventure in regard to science and research. Without his guidance and persistent help this dissertation would not have been possible.

I also would like to thank my supervisory team, Associate Professor Meixue Zhou, Dr Lana Shabala and Dr Fanrong Zeng for their tremendous assistance throughout the project and for all the effort they put in to providing me with the required trainings and data interpretation.

I am forever indebted to my beloved parents who have supported me emotionally and financially from the day I decided to come to Australia to study. At the end, my main appreciation is for my husband, Masoud Rahimian who has been the most patient angel for the long nights of thesis writing. This work would not have been successfully finished without all the help and love he has given to me.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

The world population is estimated to grow 34 percent higher than today to 9.3 billion by 2050 (Lee 2011). Accordingly, to feed the growing population the global food production needs to be doubled (Taiz 2013). This goal will be difficult to achieve considering the current world land situation. Of 5.2 billion ha under agricultural cultivation worldwide, about 3.6 billion ha is suffering from erosion, soil degradation and salinization (Riadh et al. 2010) with approximately 10% of the land surface affected by salinity (Ruan et al. 2010). Irrigation has increased the salinity problem and about 11% of irrigated land (34 Mha) is affected by salinity. Some countries such as Pakistan, China, the United States and India are more affected than the others (60 percent of the total irrigated land equal to 21 Mha is affected by salinity) (FAO 2011). Sixty seven percent of Australia's agricultural lands are potentially at risk of salinity (Rengasamy 2006). Waterlogging also limits crop production with more than one third of the irrigated lands globally affected by occasional or frequent waterlogging (Donnan and Houston 1967). Agricultural lands suffering from the concurrence of both stresses occur in many parts of the world such as Australia, USA, Pakistan, India, Iran, Thailand, and Egypt. Globally, 60-80 million ha of the lands are affected moderately by both waterlogging and salinity (WBCSD 2014). In Australia, about 2.5 Mha are affected by secondary salinity (Robertson 1996), but it is estimated by hydrological modelling that it will increase to 17 Mha in 50 years (Assessment 2000). Extreme global climate changes including heavy rainfalls, reduced freshwater availability and saltwater intrusion along coastlines have adversely affected agricultural production (Solomon et al. 2007).

Cereals such as wheat, rice, maize and barley are the most important crop plants providing food worldwide. Barley is the fourth strategic crop plant amongst the cereals, not only for its processed raw material, but also its usage as a forage grain, staple food and to malt industries (Meng et al. 2016). Barley plants are known for their moderate tolerance to salinity (Munns et al. 1995) and sensitivity to waterlogging (Garthwaite et al. 2003). Waterlogging plays the major role in exacerbating the effects of secondary salinity in barley (John et al. 1977).

According to the classical view (Munns et al. 1995), plants response to salt stress occurs in two phases: the immediate and transient osmotic phase followed by the salt-specific phase. There are three main physiological constraints affecting plant growth and survival under

salinity stress namely; (i) osmotic stress, (ii) ion toxicity and (iii) oxidative stress. Adaptation mechanisms varies between species and varieties, and their impact on plant growth is depended on the intensity and duration of the stress. The main adaptation mechanisms include; Na^+ exclusion from uptake, Na^+ sequestration (intracellular and tissue specific), control of xylem loading, Na^+ retrieval from the shoot, osmotic adjustment, K^+ retention and control of reactive oxygen species production (See Chapter 2.4 for more details).

As it was mentioned earlier salinity is not the only factor threatening agricultural production. Waterlogging has affected about 10% of the world's land area (Setter and Waters 2003). Waterlogging occurs when the pores of the soil are saturated and the soil environment has become anaerobic, however at the same time, the shoot is under normal atmospheric conditions (Striker 2012). Plant productivity drops by as much as 80% under waterlogged conditions (Shabala 2011). The reduced soil gas exchange under waterlogged conditions results in significant free oxygen reduction and carbon dioxide accumulation due to microbial and root respiration (Bailey-Serres and Voesenek 2008). Hypoxia stress starts when free O_2 around the root decreases and the roots metabolism shifts from aerobic to anaerobic, leading to intense ATP synthesis constraints (Teakle et al. 2006). Waterlogging also effects soil redox potential and chemical profile leading to a decrease in some ions such as manganese (Mn^{4+}), iron (Fe^{3+}), and sulphate (SO_2^{-4}), potentially toxic metal solubility increases (Fe^{2+}, Mn^{2+}) as does the production toxic compounds as the result of microbial and plant root anaerobic metabolism (Shabala 2011). These modifications in the plant under waterlogged conditions lead to adverse membrane transport changes, a reduction in leaf water potential and stomatal conductance, the death of plant root and shoot tissue, and ultimately can lead to the entire plant's death (Barrett-Lennard 2003).

Overall, there are three main physiological constraints affecting plant growth under waterlogging conditions; oxygen deprivation, elemental toxicity and organic phytotoxins. Plants use several tolerance mechanisms to adapt to waterlogging stress such as the formation of aerenchyma, oxygen transport to roots, control of radial oxygen loss, anaerobic metabolism of the roots, control of ROS signalling and homeostasis and mechanisms for dealing with elemental toxicity and organic phytotoxins (See Chapter 2.5 for more details).

Most plant species survival is decreased under the combined stress of waterlogging and salinity compared to waterlogged non-saline conditions and drained saline conditions (Malik et al. 2009; Colmer and Flowers 2008; Teakle et al. 2007; Barrett-Lennard 2003). Low O_2

availability under waterlogging conditions causes respiration to change from aerobic to anaerobic which leads to reduced energy production (2 instead of 36 moles of ATP per mole of hexose) (Marschner 1995a). While essential salinity tolerance mechanisms such as Na^+ exclusion at the root level and maintenance of K^+ at the shoot level are both highly energy-dependent (Tester and Davenport 2003). It has been hypothesised that the mechanisms providing better O_2 transport to overcome waterlogging stress such as the formation of aerenchymatous nodal roots leads to improved Na^+ exclusion and salt resistance of wheat in saline-waterlogged conditions (Saqib et al. 2005) by maintaining sufficient O_2 for active Na exclusion.

Generally, under combined waterlogged and saline conditions, the Na^+ and Cl^- concentration is increased and K^+ concentration is decreased compared to drained conditions (Barrett-Lennard and Shabala 2013), disturbing cell metabolism. To deal with the issue, plants adopt several strategies. Aerenchyma formation helps the plant to avoid hypoxia and maintain sufficient energy (ATP) to exclude toxic Na^+ ions. The formation of endodermis will contribute to the regulation of ion uptake and transport and reduce uncontrolled salt accumulation in the shoot. A reduction in stomatal conductance lessens the amount of saline water entering the plant. Last, salt removal strategies will help with metabolism protection (Barrett-Lennard 2003)

Even though the tolerance mechanisms of barley plants to each of above stresses are widely studied, there is not much information on the interaction of waterlogging and salinity. The severity of each component under combined waterlogging and salinity stress and their effects on plant growth and survival still remains unknown. It also remains to investigate if the stress effect is synergetic or additive. The ion relations under each stress separately is well understood but their role in plant growth under combined stresses and also the major role of each specific ion such as Na^+ and K^+ in the shoot and root needs to be studied. For the purpose of breeding, it is necessary to identify genes controlling specific traits for each stress separately and if any genes control responses to the combined actions of both stresses.

Overview

This thesis consists of seven chapters. Chapter 1 discusses the overall combined waterlogging and salinity problems and background followed by the objectives of the study.

In chapter 2, the importance of two major stresses affecting the crop production including waterlogging and salinity and their global picture in agriculture and economics is reviewed. Then major constraints imposed by each stress on the plant growth and the tolerance mechanisms are described in detail. The current knowledge of the physiological mechanisms mediating plant adaptation interaction to salinity and waterlogging is explained.

Chapter 3 provides details of the materials and methods of three sets of experiments conducted in this study. First, a whole plant study in soil under glasshouse conditions, second, second a whole shoot and root study under hydroponic conditions and finally, describing the microelectrode ion flux estimation (MIFE) technique, protocols and measurements.

In chapter 4, describes the individual and combined effects of waterlogging and salinity on whole plant agronomical and physiological characteristics of 12 glasshouse-grown barley varieties are studied ("pot experiment").

Chapter 5 describes the separate and combined effects of waterlogging and salinity on the root ionic relations under hydroponic conditions.

Chapter 6 describes the separate and combined effects of waterlogging and salinity on net ion fluxes from mature root zone of selected barley varieties.

Chapter 7 summarises the major findings of this work and highlights future directions.

Chapter 2: Literature Review

2.1 Salinity and Waterlogging: Two Major Stresses Affecting Crop Production

The world population is expected to increase to 9.3 billion by 2050, 34 percent higher than today (Lee 2011). A primary concern of growing population is the greater demand from food and less productive lands to grow it on. In order to feed the growing population by 2050 global food production is required to be doubled (Taiz 2013).

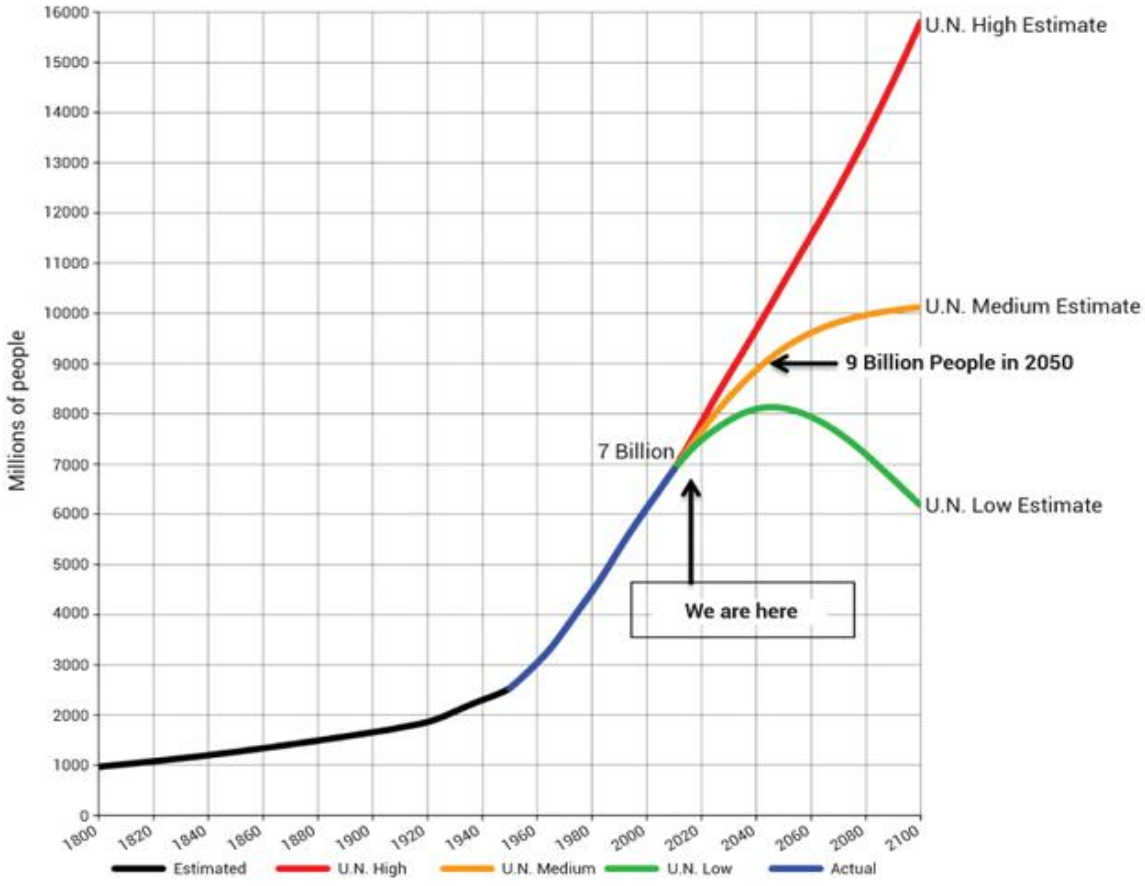

Figure 2.1. U.N. Projections of human population growth to 2100. Recent valuation; Food production is required to be doubled by 2050 to keep speed with growing population (U.N. 2011).

At the same time, about 3.6 billion of the world's 5.2 billion ha dryland under agricultural cultivation suffers from erosion, soil degradation and salinization (Riadh et al. 2010). Worldwide, about 10% of the land surface (950 Mha) is affected by salinity (Ruan et

al. 2010). Even though irrigation has played a major role in increasing agricultural production in the world, it had negatively affected the land by increasing salinization and waterlogging (FAO 2011). It is estimated that 34 Mha (11 percent of all irrigated area) are affected by some level of salinity; Pakistan, China, the United States and India are the most affected countries by 21 Mha (60 percent of the total) (FAO 2011). An additional 60–80 Mha of irrigated land are affected to some extent by waterlogging and related salinity (FAO 2011). In Australia, 67% of agricultural land has a potential for transient salinity (Rengasamy 2006). In Western Australia alone, salinity has affected more than 51% of all farms. It is estimated that 1.5 million ha of arable land are lost annually due to salinity in the world (Dulloo et al.). Annually, global agricultural production loss from salt-affected land exceeds US$12 billion and this rate is increasing (Qadir et al. 2008; Flowers et al. 2010). In Australia, the farming economy is losing A$1330 million per year to salinity (Rengasamy 2002).

Salinity is not the only stress limiting crop yields; more than one third of the world's irrigated lands also suffers from occasional or frequent waterlogging (Donmann and Houston, 1967). Irrigation is an important component in agriculture and plays a vital role in improving crop performance, but it can cause side effects such as salinization and waterlogging. Poorly managed irrigation can release salts that already exist in the soil or it can bring new salts from the water or fertilizers. While irrigation is one of the main reasons for salinization, it cannot be abandoned as 'unsustainable practice', as irrigated lands provide one third of the world's food while they take only 15% of the total cultivated lands (Munns 2005). Waterlogging is a problem linked to salinity; it reduces the available air in the soil and in turn plant growth (Dulloo et al. 2013). Waterlogging can lead to salinization as it brings the salts to the surface and stresses the plants. In many parts of the world including Australia, USA, Pakistan, India, Iran, Thailand, and Egypt these two environmental stresses are combined.

Amongst the cereals barley is the fourth largest crop after wheat, rice and maize. Barley is used in processing, as a forage grain and staple food; it has also been used in malt industries for beer, feed barley and also animal husbandry industries (Meng et al. 2016). Barley is classified as relatively salt tolerant (Munns et al. 1995) and is therefore cultivated widely around the world. At the same time, it is highly sensitive to waterlogging (McDonald et al. 2001; Garthwaite et al. 2003) Although the physiological and molecular mechanisms of barley responses to both waterlogging and salinity stresses have been studied in detail, little information is available about their combined effects. Filling these gaps is one of the aims of this work.

2.2 Salinity stress: physiological constraints

Although high levels of salinity affect plant growth, the extent of damage to the plant differs between species and even varieties. As most crop plants are glycophytes, they will not tolerate a salt concentration of more than 100-200 mM. Yield is severely limited under greater concentrations, and most tolerant species will dye when exposed to higher salinity levels. Glycophytes low tolerance to salinity is related to their development under non-saline (or low-saline) lands where they were not exposed to salt (Munns and Termaat 1986). However, halophytes were adapted to high salt concentration during their evolution and were developed to grow under sever saline conditions (more than 300-400 mM NaCl).

The classical view is that plants respond to salt stress in two phases. The first one is the *osmotic* phase and the second is *salt-specific* phase (Munns et al. 1995). In the first phase, plants growth, either sensitive or tolerant to salinity, is reduced due to the osmotic effect in the root media. In the second phase, old leaves of sensitive plants start falling down due to specific Na^+ or Cl^- toxicity effects, reducing the photosynthesis capacity of the plant.

2.2.1 Osmotic stress

Osmotic stress is the first and immediate response of the plant exposed to saline media. The term saline refers to the condition in which electrical conductivity of the saturation paste extract (ECe) goes beyond 4dSm-¹ (Richards 1954), or 40 mM NaCl. This amount of salt concentration in the soil creates an osmotic pressure of about 0.2 MPa and strikes the ability of the plant to absorb water (Shabala 2012). Osmotic pressure is a colligative property that indicates how much solute is dissolved in water.

Immediately after the salt concentration in the rhizosphere increases, the osmotic phase starts. This sudden increase in salt concentration happens together with cell water loss and a temporary decrease in the cell volume and turgor. The plant cells recoup their volume and turgor with the osmotic adjustment mechanism, even though the cell's elongation will not increase (Munns and Tester 2008). Therefore, plants under the salinity stress will have smaller and thicker leaves. Shoot growth reduction is more than root growth reduction under salt-infused osmotic stress, as plants aim to decrease the shoot water use to increase the salt tolerance (Munns and Tester 2008).

2.2.2 Ion toxicity

In many plants, salt toxicity is linked with an over-accumulation of Na^+ and Cl^- in the shoot which can cause several osmotic and metabolic problems for plants, even though the evidences for barley suggest that this plant is more sensitive to Na^+ (Møller and Tester 2007; Tester and Davenport 2003; Munns 2002). Plants exposed to saline conditions will accumulate Na^+ ions due to the strong driving force for its entry, while the plant root tends to maintain persistent levels of Na^+. Therefore, Na^+ levels in the root are regulated by exporting it back to the soil or sending it to the shoot. The rapidly moving transpiration stream in the xylem transports Na^+ from the root to the shoot and it only can be returned to the root by the phloem, which has not been proved yet (Tester and Davenport 2003).

The presence of high concentrations of Na^+ affects other ion functions negatively as well as the cytosol ionic balance. In both physical and chemical terms, Na^+ is the closest to K^+ (i.e. ionic radius and ion hydration energy). K^+ is one of the major active ions in protein synthesis and ribosome functions and it is also responsible for activation of over 50 cytoplasmic enzymes (Bhandal and Malik 1988). High volumes of Na^+ around the root leads to Na^+ replacing K^+ in vital bindings of dynamic processes in the cytoplasm, such as tRNA to ribosomes (Blaha et al. 1999) and possibly other features of ribosome function (Marschner 1995b). As a result, metabolism is severely affected and this in turn effects both root and shoot functioning. K^+ will be the most affected ion but other ions such as calcium, nitrogen, phosphorus and magnesium also are affected (Shabala and Cuin 2008)

2.2.3 Oxidative stress

Oxidative stress is defined as a multifaceted chemical and physiological phenomenon that is developed through overproduction of reactive oxygen species (ROS). It is engaged in almost all biotic and abiotic stresses in higher plants (Demidchik 2015). There are different definitions for oxidative stress in plant biology. First of all, it is a physiological condition when oxidation (loss of electrons) overreaches reduction (gain of electrons). Subsequently, the cell enzymes and structural components will be affected negatively. Second, oxidative stress is basically a stress factor that can injure the cells and cause signaling and defense reactions. Oxidative stress coupled with O_2 activation makes this molecule more active or reactive, which defines the stress caused by toxic effects of ROS (Demidchik 2012).

Salinity *per se* or in combination with other abiotic stresses, disrupts photosynthesis and increase photorespiration, modifying the regular homeostasis of cells and leads to an

increased production of ROS (Miller et al. 2010). ROS, such as $^1O_2$, H_2O_2, $O_2\cdot$- and $HO\cdot$, are known as toxic molecules that are able to cause oxidative damage to proteins, DNA and lipids (Apel and Hirt 2004). Damaging effects of ROS are because of their ability to cause lipid peroxidation in cellular membranes, DNA damage, protein denaturation, carbohydrate oxidation, pigment breakdown, and an impairment of enzymatic activity (Noctor and Foyer 1998; Scandalios 1993)

2.3 Plant adaptation to salinity

Early studies by biochemists on halophytes enzymes found that they are not much more tolerant compared to non-halophyte enzymes. The extracted enzyme of *in vitro* experiments on two halophytes, *Atriplex spongeosa* and *Suaeda maritima* showed the same level of sensitivity to salinity as non-halophytes like beans and peas (Greenway and Osmond 1972; Flowers et al. 1977). Therefore, halophyte tolerance to salinity as well as some other tolerant glycophytes species is explained by the mechanisms that the plant takes to avoid the toxic effects of NaCl. Two main mechanisms of salt tolerance are known. One includes minimizing the salt entry into the plant; the second is removing of salt from the metabolic active sites within the cytoplasm after it has entered the plant. Halophytes benefit from both mechanisms but glycophytes vary in their ability to use each or both mechanisms. Glycophytes basically are ranked from sensitive to tolerant based on their ability to use the above mentioned mechanisms.

2.3.1 Na^+ exclusion from uptake

Several studies have emphasized the role of Na^+ permeable transporters for Na^+ influx into the roots (Amtmann, Laurie et al. 1997, Roberts and Tester 1997, Tyerman, Skerrett et al. 1997). Na^+ influx to the root increases the cytoplasmic Na^+ concentration and leads to toxicity (Kingsbury and Epstein 1986). The four identified pathways for Na^+ uptake are as follows:

1. NSCC (non-selective cation channels catalyse) passive fluxes of cations through plant membranes (Demidchik and Maathuis 2007). NSCCs are permeable for K^+, Na^+ and Ca^{2+} (Demidchik 2007) and known as a main route for Na^+ uptake to the root. These channels activation is affected by various factors like cytosolic or external Ca^{2+} and pH level, cyclic nucleotides, ATP, glutamate, ROS and mechanical pressure on membrane (Demidchik and Maathuis 2007). Studies on the

Arabidopsis genome currently show 40 different NSCCs that may differ dramatically in their gating properties. It was suggested that the varieties tolerance to salinity differs based on the population of their NSCC and their sensitivity to ROS (Wu et al. 2014)

2. HKT (high-affinity Na^+/K^+- permeable transporters) The Trk/Ktr/HKT transporters probably were developed from simple K^+ channels KcsA. HKT transporters are responsible for mediating Na^+ -uniport or Na^+ /K^+ -symport and maintaining K^+ /Na^+ homeostasis to improve salinity tolerance (Su et al. 2015)

3. The low affinity cation transporter, LCT. Although this transporter has been reported many times in wheat species, it most likely is less affective for Na^+ entry to the root compared to other pathways (Amtmann et al. 2001)

4. A bypass flow, resulting from Na^+ leakage into the root via apoplast. The leakage along the transpirational bypass flow to the xylem causes one-third of the ions to reach the shoots in rice (Faiyue et al. 2010). Casparian bands at the endodermis block the ions movement in the apoplast which is not a complete blockage (Gong et al. 2006). It is noted that endodermis contains passage cells in rice roots and is permeable to Na^+ ions. Endodermal integrity and ion block leakage to the stele is improved by the silicon deposition (Gong et al. 2006). Likewise, the bypass flow of rice may be reduced three fold by Ca^{2+} under 200 mmol/L NaCl. This bypass flow reduction is positively linked with the concomitant reduction in the shoot Na^+ uptake (Anil et al. 2005)

Apart from the different roles of these pathways and reactions in different environmental conditions, they mostly illustrate very weak control of unidirectional influx of the Na^+ (Tester and Davenport 2003). Studies show there is not a big difference in root Na^+ content of varieties with high or low tolerance to salinity. It was also found that the amount of Na^+ accumulated in the roots is much bigger than the shoots. What we now know about the Na^+ uptake in the roots is that a big volume of Na^+ (up to 95-97%) is moved back to the rhizosphere before being transported to the shoots (Munns 2002).

2.3.2 Na^+ Sequestration

2.3.2.1 Intracellular Sodium Sequestration

Cellular Na^+ sequestration to the vacuole is essential for plant for two main purposes: (1) to achieve osmotic adjustment and (2) to avoid the toxic effects of high amounts of Na^+ in the cytosol. Na^+ sequestration to the vacuole is energetically the most efficient way to achieve osmotic adjustments under saline conditions. The required energy for pumping 1 mol of Na^+ against the electrochemical gradient into the vacuole is 1/10 of the total energy required to produce 1 mole of organic osmolyte (equal to 3.5 mol of ATP) to maintain the osmotic balance of the cells (Shabala and Shabala 2011). As detailed in section 2.3, there is little, if any difference, between the tolerance of cytosolic enzymes of halophytes and glycophytes (Flowers and Colmer 2008). Therefore, it is absolutely essential to keep cytosolic Na^+ content at non-toxic levels.

Involvement of NHX antiporters in these processes is indicated by the induction of Na^+/H^+ antiport activity or NHX gene expression in aerial parts or roots of many plant species when grown in saline environments (Rodríguez-Rosales et al. 2009). A gene coding for a Na^+/H^+ exchanger (NHX1) was initially recognized by silico analysis of the yeast genome (Nass et al. 1997). Afterwards, it was shown that under saline conditions, NHX1 was responsible for prevacuolar/vacuolar compartmentalization of Na^+ ions (Nass and Rao 1998). Na^+/H^+ antiporters exchange protons for Na^+ through vacuole membrane in plants, algae and fungi (Adem et al. 2015). NHX1 located in the plants vacuole removes Na^+ from the cytoplasm. This process is energized by the electrochemical potential created by the pumping of H^+ into the vacuole which is applicable by two proton pumps, vacuolar H^+-inorganic pyrophosphatase (V-inorganic pyrophosphatase [V-PPase], and vacuolar H^+-ATPase (V-adenosine artiphosphatase [V-ATPase] (Adem et al. 2015)

2.3.2.2 Tissue Specific Sodium Sequestration

Younger tissues always contain lower amounts of salt concentration which is more obvious when the new leaves are growing and enlarging in cell size. It happens because of the phloem of the growing tissues and as phloem contains a very small amount of salt concentration and when they are fully grown they have been transpiring for a shorter time (Shabala 2012).

The most susceptible part of the leaves are their blades and they may accumulate salt to possibly toxic levels. The two main reasons for this are; first, the transpiration stream ends

there and second, there are restrictions for the salt exported in the leaves. In many species, Na^+ can be retrieved from the xylem and flows through a leaf base and goes in direction of the tip; or flows through an elongated stem and goes in direction of shoot apex. In monocotyledonous species, such as barley, Na^+ can be retrieved and stowed in leaf sheaths (Davenport et al. 2005). Salt removal from xylem channels decreases salt flux into young leaves and leads to considerable deposition of Na^+ in stem tissues.

Na^+ transport to photosynthetic tissues has to be restricted to maintain the plant tolerance to salinity (Munns 2002). If Na^+ transport and xylem loading to the shoot is not controlled, Na^+ toxic levels will be collected in the leaf blade, resulting in the impairment of metabolic functions (Maathuis and Amtmann 1999).

2.3.3 Control of xylem loading

Regulating Na^+ xylem loading plays a major role amongst various salinity tolerance physiological mechanisms in the plant (Munns and Tester 2008; Tester and Davenport 2003). The considerable amount of entered Na^+ into the root in halophytes is transported to the shoot via the transpiration stream as a low-cost osmoticum to retain cell turgor. Na^+ xylem sap content could be about 50mM in halophytes (Shabala and Mackay 2011). High xylem Na^+ concentration will also help with water transport to the shoot through the formation of water potential gradients (Balnokin et al. 2005; Shabala et al. 2013). Xylem Na^+ loading is highly dynamic and changes dramatically during stress. At the best situation in halophytes, immediately after salt stress onset, the required amount of Na^+ is sent to the shoot to attain the full osmotic adjustment and retain the normal growth rate. When the osmotic adjustment is achieved, the plant is required to minimize xylem Na^+ loading to just provide cell turgor in newly growing tissues. So, salinity tolerance seems to be dependent on timing of the xylem Na^+ loading regulation (Shabala 2013). In glycophytes, barley as a salt-tolerant species quickly loaded Na^+ in the xylem within the first 6 hours of salt stress onset and kept the xylem Na^+ stable for four weeks of the experiment. At the same time, salt-sensitive pea plants attempted to strictly control the xylem Na^+ loading immediately after the stress onset but failed to maintain it a long term (Bose et al. 2014). Salt-sensitive species such as pea plants approach the Na^+ exclusion, and therefore do not keep much Na^+ for osmotic adjustment. Slow but steady Na^+ delivery to the shoot, simultaneously with its active exclusion from the mesophyll results in an immense Na^+ volume in the apoplast, above 500mM after 4 weeks of salt stress (Bose et al. 2014). Such massive Na^+ concentrations leads

to severe osmotic stress on the leaf mesophyll and also immense plasma membrane depolarization resulting in K^+ leakage from the mesophyll (Shabala et al. 2000). Cytosolic K^+ homeostasis disruption will activate caspase-like proteases and endonucleases (Demidchik et al. 2010; Shabala et al. 2007; Hughes Jr and Cidlowski 1999) which lead to cell death. It is concluded that plant salinity tolerance is highly affected by time-dependent regulation of the rate of xylem Na^+ loading.

The xylem loading process is controlled by several cation and anion transporters (Shabala and Mackay 2011; De Boer and Volkov 2003). Xylem Na^+ loading in plants can happen through passive or active transport systems. First, xylem Na^+ loading can happen through passive channel-mediated processes. The nominated channel for xylem–parenchyma interface in glycophytes is Na^+-permeable non-selective outward-rectifying channels (NORK) (Wegner and De Boer 1997; Wegner and Raschke 1994). NORK activation is controlled by several factors under saline conditions like apoplastic pH (Lacombe et al. 2000), polyamines (Zhao et al. 2007) and abcisic acid (Pilot et al. 2003). Second, Na^+ might be loaded into the xylem through thermodynamically active xylem Na^+ loading mechanism (Shabala and Mackay 2011; De Boer and Volkov 2003); two possible candidates are SOS1 Na^+/H^+ exchanger and cation-Cl (CCC) co-transporter. Most likely, both passive and active pathways are involved, but their corresponding function may vary based on the stress duration. Figure 2.2 shows a hypothetical model illustrating xylem Na^+ loading.

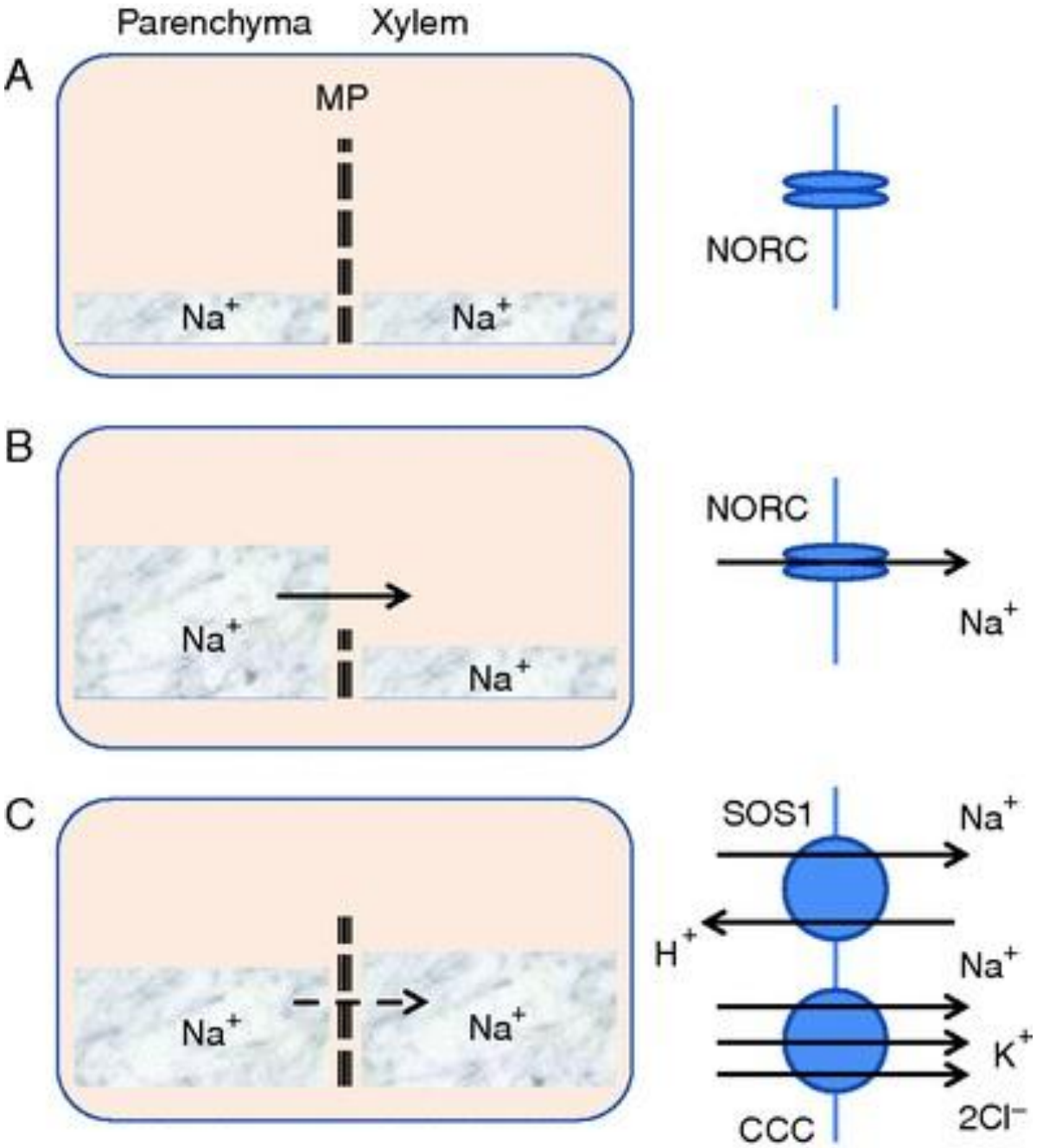

Figure 2.2. A hypothetical model representing xylem loading kinetics and the mechanisms involved. Before onset of salinity stress (A), passive xylem Na+ loading via non-selective cation channels (NORCs) is not possible due to low cytosolic Na+ concentrations in xylem parenchyma and negative membrane potential. After applying salinity stress (B), root cells will be depolarized (Wegner et al. 2011), alongside with progressive accumulation of Na+ in the parenchyma cell cytosol. At the same time, low xylem Na+ concentration enables channel-mediated xylem Na+ loading. Xylem Na+ concentration increases by time and parenchyma cells become repolarized, therefore further passive loading is not feasible. Two active transporter may be responsible for further xylem Na+ loading: SOS1 ((Na+/H+ exchanger) or CCC (2Cl−:Na+:K+ symporter) (C) (Shabala 2013) .

Two main pathways help to reduce Na^+ content in the xylem: First, minimizing Na^+ entry to the xylem from the root symplast. Second, maximizing Na^+ retrieval from the root before it is loaded to the sensitive tissues in the shoot (Flowers and Yeo 1992).

2.3.4 Na^+ retrieval from the shoot

Reduction in Na^+ accumulation is important for plant tolerance, therefore Na^+ content of xylem is needed to be removed before reaching the bulk of the shoot (Tester and Davenport 2003). Three suggested main 'checkpoints' for Na^+ removal are , root mature zone, shoot vasculature and, the base of the shoot (Shabala 2012).

Reducing the rate of Na^+ transport from roots to shoots is conferred by Nax_2 locus by retrieving Na^+ from the root xylem (Davenport et al. 2005; James et al. 2006). Fine mapping of Nax_2 locus in wheat was not possible because of the shortage in recombination in the chromosomal region. Hence, nominee genes that could impart Na^+ exclusion were recognized by screening for genes that confer comparable phenotypes in several other species (Byrt et al. 2007). HKT (high-affinity Na^+/K^+-permeable transporters) is known to be able to contribute to Na^+ exclusion from leaves by retrieving Na^+ from the xylem when they are expressed in the xylem parenchyma cells (Munns and Tester 2008; Horie et al. 2009). The proteins that are encoded by group 1 HKT genes of *Arabidopsis thaliana*and rice, *AtHKT1;1* and *OsHKT1;5*, decrease transport of Na^+ to shoots and leads to increasing plant salinity tolerance (Horie et al. 2005; Plett et al. 2010; Ren et al. 2005; Møller et al. 2009; Davenport et al. 2007; Mäser et al. 2002).

2.3.5 Osmotic adjustment

Plants under salinity stress suffer from osmotic stress and losing cell turgor. The osmotic effect of salinity happens immediately after salinity onset and are reported to dominate for a few weeks (Munns 2002). Osmotic adjustment is one of the tolerance mechanisms to rescue plants. Shoot cells are required to keep positive turgor under hyperosmotic conditions in the rhizosphere to maintain leaf expansion growth which needs osmotic adjustment. There are three main paths to gain osmotic adjustment (Shabala 2011). First, taking the organic osmolytes (compatible solutes); second, *de novo* synthesis of compatible solutes and third, accumulating inorganic osmolytes such as K^+, Na^+ and Cl^- instead of organic osmolytes.

2.3.6 K^+ retention

The large amount of Na^+ in the cytosol and its toxic effect does not rely on only Na^+ exclusion. Plants with good features of tolerance retain K^+ to regulate cytosolic K^+/Na^+ ratio to prevent replacing K^+ with Na^+ under saline conditions to help with enzyme activation and protein biosynthesis (Shabala and Cuin 2008). Intracellular K^+ homeostasis regulation is necessary to mediate plant adaptive responses to a wide range of abiotic and biotic stresses such as salinity. It is established that K^+ is not only an essential nutrient for plant growth and increasing the yield but also a main signaling agent mediating a broad range of plant adaptive responses to the environment (Anschütz et al. 2014). K^+ retention is an important factor for salt-tolerance which was studied during the last decade in both root (Shabala and Cuin 2008) and shoot mesophyll. Salt-tolerant barley varieties under saline conditions showed higher K^+ concentration in roots compared to salt-sensitive varieties under the same conditions (Liang 1999). A strong positive correlation was found between root K^+ retention and plant salinity tolerance under saline conditions for several species including barley (Chen et al. 2007b; Chen et al. 2007a; Chen et al. 2005). A positive correlation was reported between leaf mesophyll K^+ retention (calculated by the magnitude of NaCl-induced K^+ efflux from mesophyll) and overall salinity tolerance (relative fresh weight and/or survival or damage) for 46 barley varieties under saline conditions (Wu et al. 2015).

2.3.7 Control of ROS production

The salt stress is a major reason for the accumulation of ROS. The controlled ROS production has key role in plant growth and development. But when ROS production exceeds the limits, the subsequent uncontrolled oxidation leads to cellular damage and ultimate cell death (Noctor and Foyer 1998). The capability of plants to manage the generation of ROS is a vital aspect of tolerance as ROS causes irrevocable damage to plants by disrupting cellular homeostasis. The concentration of ROS under normal conditions is controlled by an array of enzymatic and non-enzymatic antioxidants. These antioxidants induce a high level of stress tolerance in plant system.

Catalase (CAT) and ascorbate peroxidase (APX) are the two major enzymes involved in H_2O_2 detoxification. CAT catalysis the dismutation of H_2O_2 to H2O and O_2 while APX catalysis the formation of monodehydroascorbate by consumption of H_2O_2 thereby, protecting the plant from its deleterious effects. There are some other enzymatic controllers such as: Superoxide dismutase (SOD) that dismutates $O_2 \cdot -$ into H_2O_2 and is suggested to act

as the 'first line of defence against oxidative stress in plants (Alscher et al. 2002); Peroxidase (POX) from the isoenzymes family that are scavenging H_2O_2 mostly in the apoplastic space (Fagerstedt et al. 2010) and Redox regulatory enzymes that perform as antioxidants in both glycophytes and halophytes(Ozgur et al. 2013).

There are highly toxic ROS such as $1O_2$ and $OH\cdot$ that cannot be scavenged by enzymatic antioxidants. The main non-enzymatic antioxidants that halophytes are dependent on to scavenge these specific ROS are: ascorbate, glutathione, glycine-betaine, proline, polyamines, tocopherols, polyols, iron-binding proteins, carotenoids, polyphenols and sulphated polysaccharides (Bose et al. 2013)

2.4 Waterlogging stress: physiological constraints

The term waterlogging originates from a wider definition of flooding that represents different situations from water saturated soil (waterlogging) to submerged plants. The term waterlogging is referring to the situation when the whole root system of plants is under anaerobic condition. This occurs when all the pores of the soil are saturated with water and the soil surface is covered by a very thin layer of water, while the shoot is still under the atmospheric normal condition. When the water layer above the soil surface covers parts or the whole shoot, it can be defined as partial or complete submergence of the plants (Striker 2012).

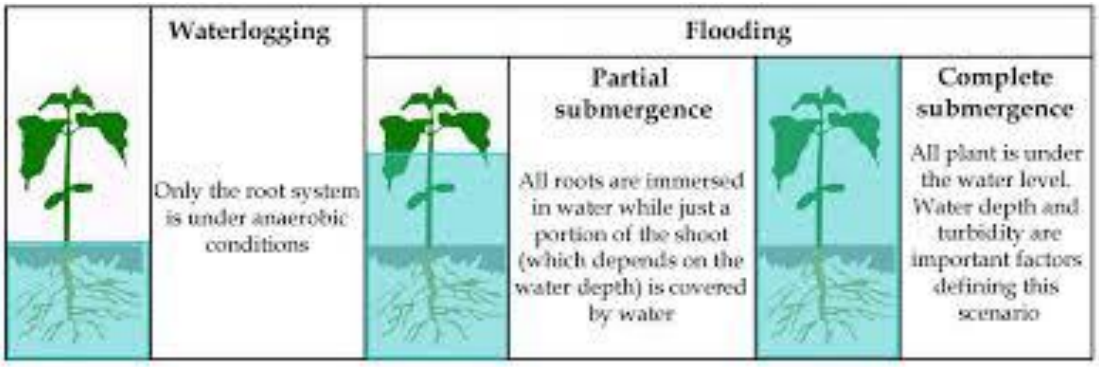

Figure 2.3. Waterlogging is defined by the time the soil is saturated with water and soil surface is covered by a very thin layer of water. Flooding divides to two groups: when part (partial submergence) or whole (complete submergence) the shoot is covered by water (Striker 2012)

The presence of excess water in the root zone of the plants called waterlogging results in inadequate exchange of gases. The excess of water after evaporation leads to accumulation of salt near the root zone and it reduces the potential yield of salt-sensitive crops by more than ten percent (Moore and McFarlane, 1998).

The increase in the magnitude of waterlogging is associated with high precipitation especially in high and mid-latitude regions (García et al. 2007). Hypoxic conditions arise in waterlogged soil due to low diffusivity of oxygen in pores filled with water and already present dissolved oxygen being utilized by roots. With time waterlogging leads to the accumulation of ethylene, carbon dioxide, ethanol and lactate which are anaerobic bacterial metabolites and also reduction in redox potential of soil due to consumption of nitrate, ferric and sulfate ions (Striker 2012)

Waterlogging severely reduces yield production of aerobic crops by 20–50% (Zhang et al. 2006). Since anthesis and maturity periods are critical for grain formation, waterlogging postanthesis becomes the limiting factor for wheat yield improvement in this area. Depressed photosynthesis resulting from destroyed pigments and promoting leaf senescence are the deleterious responses (Smethurst and Shabala 2003) which affect plant growth, dry matter accumulation and distribution in waterlogged soil (Brisson et al. 2002). Waterlogging becomes a severe concern when the soil is not levelled and irrigation is followed by excess rain (Gill et al., 1992). Large waterlogged areas of southern and south-east Asia are utilized for the crop rotation of rice with wheat. The compaction of subsoil occurs due to optimized flooding conditions required for rice cultivation exposing wheat to waterlogged conditions (Samad et al., 2001). One of the major causes of waterlogging is the irrigation of the fine textured soil with water containing high carbonate and bicarbonate levels which induces sodicity (Quereshi and Barrett-Lennard, 1998). The transient waterlogging conditions occurs in sandy soils of Australia where rainfall quickly penetrates sandy topsoil and accumulates above compact clay subsoils (Zhang et al. 2006).

The main physiological constraints affecting plants growth under waterlogging conditions are discussed below.

2.4.1 Oxygen deprivation

Plants rooting in overflowed soils need to adapt to an absence of oxygen, or anaerobiosis. Respiration (oxidative phosphorylation) as the most proficient fountain of energy is

responsible for root growth and sustenance. Respiration needs adequate O_2 concentration which differs among species and varieties ("Pasteur point"; ca. 0.2%) and when it drops beneath a certain concentration is totally restrained. Respiration will still be active even at a reduced rate under lower than critical oxygen pressure (COPR; lowest concentration to maintenance maximum respiration rate; (Armstrong, Webb et al. 2009)). At the point when respiration is lessened at low O_2 supply (hypoxia) or in lack of O_2 (anoxia), root cells have the capacity to transform to fermentation for ATP synthesis (Thomson and Greenway 1991). Nevertheless, respiration is thermodynamically more efficient than fermentation. While respiration synthesizes 36 molecules ATP per mole glucose, fermentation produces only about 3 molecules (Wegner 2010). Continued oxygen deprivation results in accretion of ethanol in tissues that can reach a toxic level. Toxicity can be produced also by bacteria that occur under anaerobic conditions (Bailey-Serres and Chang 2005; Triantaphylidès et al. 2008).

2.4.2 Elemental toxicity

Most plants are negatively affected by waterlogging stress. Soil microbes, in shortage of oxygen, use NO^{3-}, Fe^{3+} and Mn^{4+} as an alternative for electron acceptors of O_2, leading to highly chemically reduced conditions. SO_4^{2-} and CO_2 are the next alternatives under severe waterlogging conditions (Madigan et al. 2003). Some plant nutrients such as nitrogen are less available under reduced conditions (Zhang et al. 1990), while the availability of some micronutrients such as Fe and Mn are intensely improved (Plekhanova 2007). Fe solubilization also leads to increased phosphorus availability under waterlogging conditions (Gambrell and Patrick Jr 1978)

Waterlogging reduces the availability of some essential nutrients like nitrogen (Smethurst et al. 2005) or alter their availability such as N and S, at the same time increasing the availability of some other nutrients like Mn^{2+}, Fe and Zn. This condition also upsurges the transmission of mobile nutrients. Adaptive responses to different types of anaerobic conditions including hypoxia and anoxia depend on their duration of adaption and are still questioned. The lack of a general model can be explained by the absence of a control over the stresses applied to the plants. Plant root capacity to uptake nutrients is reduced under waterlogging while the nutrient availability is increased; but conceivably to a toxic level (reviewed by (Mancuso and Shabala 2010)).

Redox potential linked to the level of waterlogging changes the metabolites production. Nitrate (NO_3^-) is used as an alternative electron acceptor in respiration when the free oxygen is consumed. Around 225mV reduction/oxidation potential (correlated to pH7) during the denitrification, nitrate reduces to three main parts: nitrite (NO_2^-), different types of nitrous oxides (e.g. N_2O, NO) and molecular nitrogen (N_2) (Gambrell et al. 1991). When the redox potential drops to 200mV, manganese oxides are used as the second alternative electron acceptor. After redox potential decrease to above 100mV, ferric iron will reduce to ferrous which is more soluble and mobile. This reduction is correlated with pH increase in soil (Kirk et al. 1990). At about -150mV redox potential, sulphate reduces to sulphite. When it drops to -200mV, methane is formed from the reduction of carbon dioxide (Marschner 2011).

2.4.3 Organic phytotoxins

Besides the accumulation of inorganic phytotoxins such as Fe^{2+}, Mn^{2+} or H_2S, organic substances such as ethanol, acetaldehyde and various short-chain fatty acids and phenolics are also accumulated under waterlogging conditions significantly (Shabala 2011). Reduction/ oxidation potential of the soil reduces instantly after waterlogging. Subsequently, due to anaerobic metabolism in both plants (Lynch 1977; Armstrong and Armstrong 1999) and microorganisms (Drew and Lynch 1980), the mentioned organic substances are produced as secondary metabolites (Voesenek et al. 1999).

2.5 Plant adaptation to waterlogging

2.5.1 Aerenchyma formation

In order to allow the roots to retain aerobic respiration under hypoxic or anoxic conditions, plants develop highly porous root cortex forming an aerenchyma tissue. Aerenchyma formation improves the internal dispersion of photosynthetic and atmospheric oxygen from airborne parts to waterlogged roots (Armstrong 1979). As this results in the presence of longitudinal aerenchyma, plant species can grow their roots into waterlogged soils and increase their tolerance to anaerobic conditions. Cereal crops such as barley, wheat, rice and maize usually form the cortical aerenchyma (i.e., primary aerenchyma) in the roots. Most legume crops form a white, spongy tissue filled with gas space (secondary aerenchyma) in the stem, hypocotyl, tap root, adventitious roots and root nodules under waterlogging conditions (Yamauchi et al. 2013)

There are two types of primary aerenchyma based on their formation including *schizogenuous* aerenchyma and *lysigenuous* aerenchyma (Evans 2004). Schizogenous aerenchyma grows gas spaces over cell division with distance from the apex without cell death. Lysigenous aerenchyma, by contrast are shaped by programmed cell death and following lysis of some cells in cereal crops like wheat (Trought and Drew 1980) and barley (Arikado and Adachi 1955). Formation of the aerenchyma is a constitutive trait in waterlogging-tolerant species such as rice (Kuo, 1993) but has to be induced in most other crop species.

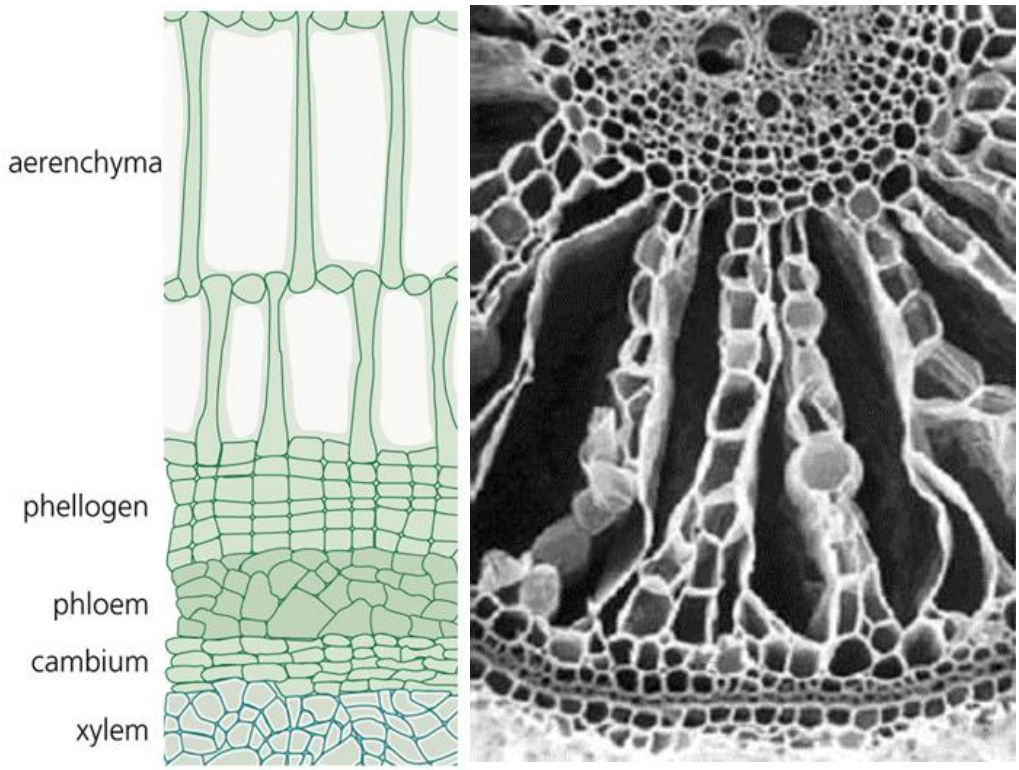

Figure 2.4. Scanning electron micrograph illustrating the aerenchyma in young rice root (Jackson 2004)

2.5.2 Oxygen transport from roots

Oxygen transport in gas-filled spaces of the plant body is regularly diffusive (Armstrong 1979), additionally different mechanisms for convective mass flow (ventilation) has also been progressed. *Diffusion* is defined as the mechanism causing gas movement into and along the plant roots (Armstrong 1979; Beckett et al. 1988). There are limiting factors for the capacity of longitudinal O_2 diffusion such as: anatomical, morphological, and physiological

characteristics, in addition to environmental conditions such as temperature and demand for O_2 in the rhizosphere (Colmer 2003).

2.5.3 Control of radial oxygen loss

When roots are exposed to waterlogged conditions, the only oxygen source is a downward transport from the shoot that remains in contact with the atmosphere. Under such conditions, minimizing the radial oxygen loss (ROL) from the root to provide adequate oxygen for root apex is vital. ROL acts as a barrier to radial oxygen loss by tissues. These barriers are activated under waterlogging stress in some species like rice (Colmer et al. 1998), *Hordeum marinum* (Garthwaite et al. 2008) and *Caltha Palustris* (Visser et al. 2000).

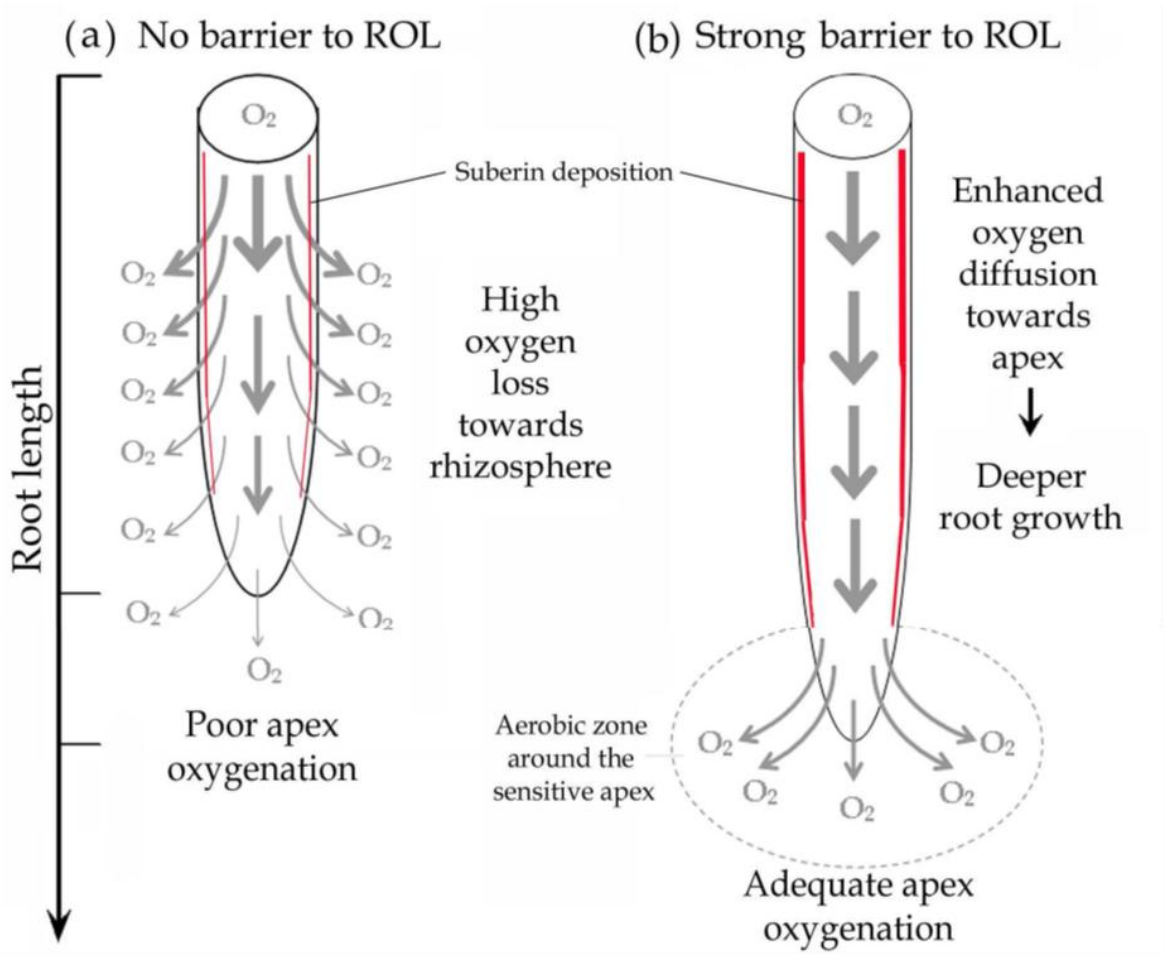

Figure 2.5. Figure representing two roots with and without radial oxygen loss (ROL). In the shown hypothetic examples, the root aerenchyma is not considered as a controlling factor for oxygen transport. Roots without barrier to ROL in the outer cortex (a) lose oxygen laterally leading to a deficient apex oxygenation and shorter roots under anoxia stress. Roots with as strong barrier to ROL (b) are able to transport oxygen efficiently to the apex leading to deeper root growth under waterlogging conditions. Suberin deposition in the cell walls of the outer root cortex and/or the exodermis results in physical barrier to ROL which is shown by

the red lines with different width in (a) and (b). The width of grey arrows illustrates the available oxygen amount (Striker 2012).

Aerenchyma in rice roots in apical and basal parts is shown in Figure 2.6. As it can be seen, aerenchyma in apical parts by itself is not practical without patterns of radial O_2 loss (ROL) under waterlogged soil.

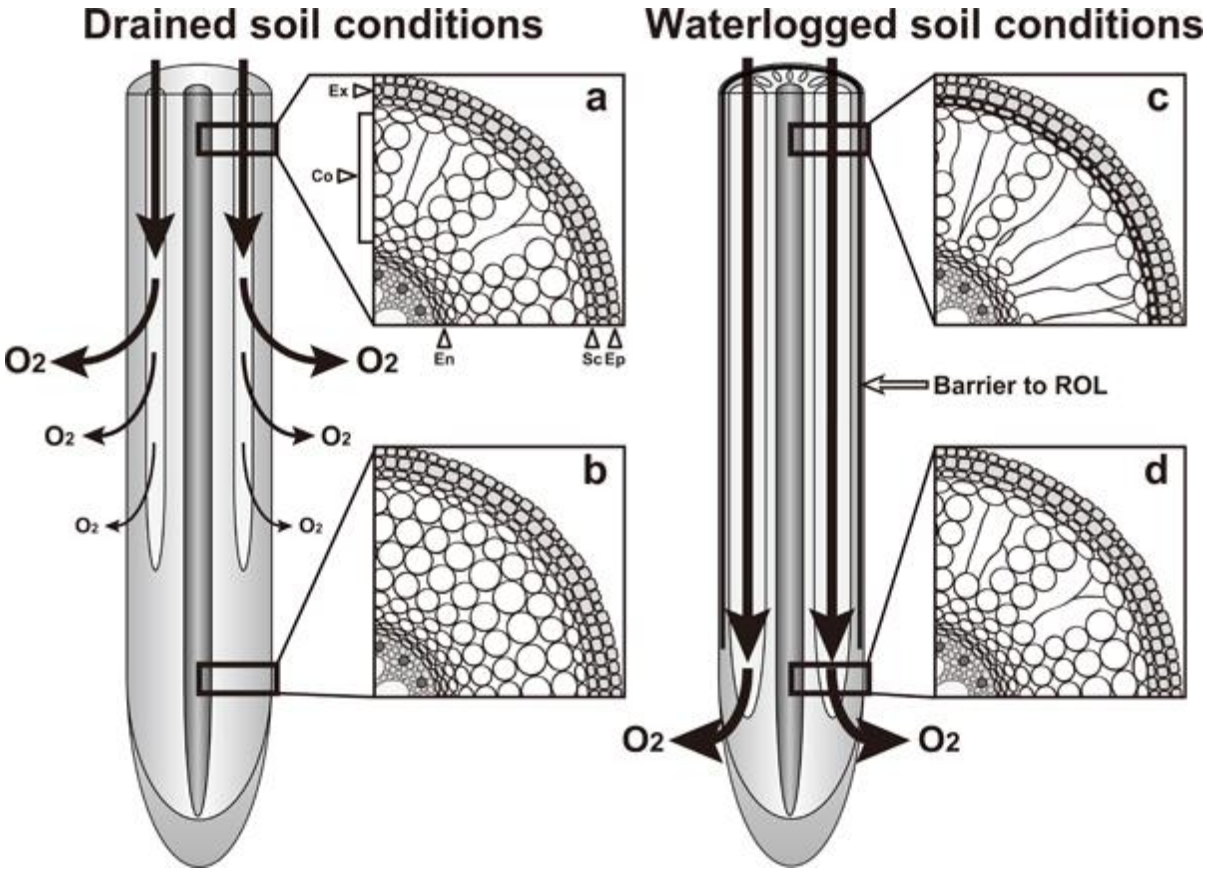

Figure 2.6. Differences in lysigenous aerenchyma formation and patterns of radial O_2 loss (ROL) in rice roots under drained soil conditions and waterlogged soil conditions. Lysigenous aerenchyma is constitutively formed at the basal part of the roots (a) even when the soil is well drained but not usually at the apical parts (b). Under waterlogging conditions lysigenous aerenchyma formation is induced at the basal part (c) and the apical part (d) of the roots. Barrier to ROL formation is only induced under waterlogged soil conditions (Nishiuchi et al. 2012)

2.5.4 Anaerobic metabolism of roots

Plants respond to anoxia by shifting the form of protein synthesis. Aerobic protein synthesis is ceased in the first hour of anaerobiosis in maize, simultaneously synthesis of a class of polypeptides with approximate molecular weights of 33,000 daltons increases. In the second hour of anaerobic stress, the synthesis of alternative small group of polypeptides is started. This small group is called the anaerobic polypeptides (ANPs) that accounts for >70%

of total protein synthesis after 5 hr of anaerobiosis in maize, and its synthesis is mostly at the same ratio until root death (~70 hr) (Sachs et al. 1980). Protein chemistry and molecular studies have recognized several ANPs that are all glycolytic enzymes (Miernyk 1990; Mujer et al. 1993). The prime ANPs that has been studied broadly is alcohol dehydrogenase (ADH) polypeptides (Sachs et al. 1980).

1. Ethanolic Fermentation

Plant root cells undertake anaerobic fermentation to accomplish the cellular demand for ATP under anoxia conditions, as oxidative phosphorylation of mitochondria is congested (Davies, 1980). To maintain the glycolysis process, ADH is subjected to NAD^+ recycling during alcoholic fermentation (Saglioet al., 1980). For the purpose of categorizing plants into flood tolerant and intolerant the formation of paired ADH with ethanol production has been studied (Crawford, 1967; McManmon and Crawford, 1971). Flood-tolerant plants showed high levels of ADH activity and ethanol production during anaerobiosis (Avadhani et al., 1978;Chirkova, 1978; Smith and ap Rees, 1979; Tripepi and Mitchell, 1984). ADH activity and flood injury index were positively correlated in different species (Francis et al., 1974; Liao and Lin, 1995; Lin and Lin, 1992). Less tolerant species had higher ethanol production compared to tolerant species of waterlogging (Barta, 1984; Crawford, 1967, 1978). The "pH Stat" hypothesis explains that ethanol is the less declining end product of fermentation rather than lactate in short-term flood tolerance (Davies, 1980). Acidification of sensitive plants to flooding like maize, wheat and barley happens by accumulation of lactate (Roberts et al., 1984, Menegus et al., 1989, 1991). Although more considerable concentrations of ethanol can be tolerated by the plants than those recorded initially (Jackson et al., 1982), plants are not able to accommodate ethanol infinitely. For example, pea seedlings can tolerate ethanol concentration of about 60mmol/L, and any concentration above that limit will lead to anoxic death. In conclusion. Therefore flooded roots need to expel the excess ethanol by aerobic metabolism to avoid poisoning (Barclay and Crawford 1981). Remarkably, due to much lower activity of PDC (pyruvate decarboxylase) compared to ADHPDC is expected to be the rate-limiting enzyme of ethanol synthesis rather than ADH (Chang et al., 1983; Su and Lin, 1996; Waters et al., 1991).

2. Alternative fermentation pathways

Crawford's theory suggests that diminishing the ethanol production leads to flooding tolerance and is correlated with re-routing from ethanol fermentation to malate production (Crawford, 1967; McManmon and Crawford, 1971). The presence of PEPC

(phosphoenolpyruvate carboxylase) and MDH (malate dehydrogenase) for malate synthesis and absence of NADP-ME (NADP-malic enzyme) for avoiding the malate decarboxylation is essential. Conversely, succinate was proposed to be the fermentation end product rather than malate (Vanlerberghe et al.1990). Vanlerberghe et al suggested a model that succinate is collected through a partial tricarboxylic acid pathway from fumarate via oxaloacetate and malate. Prolonged hypoxia leads to increased lactate dehydrogenase activity which could be a key factor in long-term adaptation to waterlogging (Hoffman et al., 1986).

2.5.5 Dealing with elemental toxicities

Plants adaptative mechanisms to elemental toxicities such as Fe and Mn are not fully known; the suggested tolerance mechanisms are as follows. Fe toxicity seems to be a genetically and physiologically multifaceted trait. There are some proposed tolerance mechanisms to Fe toxicity including Fe^{2+} oxidation in the rhizosphere, exclusion of Fe^{2+} from root uptake, storage of the excessive Fe^{2+} in the apoplast and vacuole, and detoxification of Fe-induced ROS by antioxidant enzymes (Dufey et al. 2009). Mn tolerance mechanisms are generally unknown (Wang et al. 2002), there are two suggested mechanisms to explain the plant tolerance to $Mn^{2+.}$

Mn^{2+} is prevented from entering the cytosol by an exclusion mechanism and this helps to minimize its damaging effects in the apoplast. Tissue tolerance mechanisms help plants to take up and accumulate Mn^{2+} because of complexation, detoxification and compartmentalization of Mn^{2+} within the plant. In barley, Mn^{2+} exclusion could be the primary tolerance mechanism, with most of the tolerant genotypes having low Mn^{2+} concentrations in leaves. Internal tolerance mechanisms might occur in several other Mn^{2+-} tolerant genotypes (Huang et al. 2015)

2.5.6 Dealing with organic phytotoxins

The energy metabolism under waterlogging conditions is shifted to anaerobic mode from aerobic (Gibbs and Greenway 2003). Both alcoholic and lactic acid fermentation are the major factors in anaerobic stress tolerance by retaining lower redox potential and permitting the persistence of glycolysis via pyruvate consumption and recycling NADPH to NADP (Drew 1997; Sairam et al. 2009). Nevertheless, anaerobic respiration in submerged organs such as roots possibly will also end up with the accumulation of acetaldehyde and ethanol, leading to cell death in roots and damage to shoots during waterlogging (Drew and Lynch 1980; Sairam et al. 2009). The plant tolerance mechanisms for the above mentioned stress

remains ambiguous (Shabala 2011). So far, self-poisoning by ethanol is not assumed to be the chief reason of injury in sensitive plants to waterlogging, even though the role of acetaldehyde on the ethanol-induced injuries is detailed (Perata and Alpi 1991; Perata and Alpi 1993). It is shown that tolerant species to waterlogging had lower *in vivo* conversion rate of ethanol to acetaldehyde (Kato‐Noguchi 2002).

Ethylene is the next potential toxic metabolite that was found in waterlogged soils. However, ethylene's restrictive role is unlikely to be associated with its toxicity *per se*, but rather is initiated by the disturbance to signal transduction pathways. Ethylene has been connected with control of root hairs (Petruzzelli et al. 2003), aerenchyma formation (Evans 2004), K^+ starvation responses (Jung et al. 2009) and oxidative stress signaling (Laohavisit and Davies 2007; Jung et al. 2009). It is suggested that ethylene functioning is through interaction with other hormones or signaling pathways (Laohavisit and Davies 2007).

2.5.7 ROS Signalling and Homeostasis

Anoxia conditions caused by waterlogging stress damages plant membrane structure and function leading to ROS production. ROS production results in peroxidation of lipid membranes, decreases in reduced glutathione level, an increase in cytosolic Ca^{2+} concentration, oxidation of protein thiol groups and membrane depolarization (Blokhina et al. 2003). Levels of ROS needs to be regulated to secure plant cells under stress conditions. SOD is the major factor to overcome ROS because of its chief role in cellular defense against oxidative stress, as its activity directly adjusts the amount of O_2^- and H_2O_2 (Halliwell 1987).

NADPH oxidase is a vital source of ROS in stressed plants. NADPH oxidase transfers electrons from cytoplasmic NAD(P)H to O_2 to form the superoxide radical (O_2^-) (Apel and Hirt 2004). Additionally, NADPH oxidase (NOX) plays a crucial role in plant growth and development and also in response to several abiotic and biotic stresses. NOX also performs as an ROS provider which is required for signal transduction and stress perception in plants. Nevertheless, it still remains unanswered if NADPH oxidase is involved in the combination of waterlogging and salinity stress-induced ROS production in plants (Turkan et al. 2013).

2.6 Combined salinity/waterlogging stress in nature

2.6.1 Occurrence in nature and effects on agricultural crop production

Even though waterlogging itself is an extensive stress, it can also be an extra soil restriction in landscapes affected by salinity (Barrett-Lennard 2003; Bennett et al. 2009). The combined stress occurs when salinity coincides with a shallow water table or when there is reduced penetration of surface water due to soil sodicity. Such a condition can occur in landscapes that are contiguous to estuaries and river flood plains and on agricultural lands that are affected by secondary salinity (dryland and irrigated) and also in the lands that are irrigated with water of 'high sodium hazard' (Qureshi and Barrett-Lennard 1998; Ghassemi et al. 1995).

2.6.2 Physiological limitation imposed on crops by combined WL/NaCl stress

The detrimental effects of waterlogging and salinity on the crop yield has become a global concern which enhances the significance of the research in understanding the physiology of tolerance in plants especially cereals under salinity and waterlogging. Photosynthetic response to waterlogging and salt stress is highly complex involving the interaction of different cellular pathways at different sites during plant development. The adverse interaction between waterlogging and salinity on plant survival is stated due to the decrease in survival of most of species studied under the combination of waterlogging and salinity which was greater than the product of the decrease in survival under waterlogged (non-saline) conditions and the decrease in survival under (drained) saline conditions (Barrett-Lennard 2003).

The plant response to combination of waterlogging and salinity stress is influenced by the intensity and duration of each stress. Acclimation responses for waterlogging indirectly affect photosynthesis by restricting gaseous exchange and responses to salinity also include synthesis of osmolytes as well as adjustments in ion concentration through transportation across the membrane. In most species the combined effects of salinity and waterlogging results in increased concentrations of Na^+ and Cl^- and decreased concentrations of K^+ in the shoots/leaves of plants, compared with salinity under drained conditions (Barrett-Lennard and Shabala 2013). There are two possible mechanisms underlying this phenomenon. First, there are common transport systems that are mediating the simultaneous but oppositely

directed changes in Na^+ and K^+. NSCC (non-selective cation channels) can be nominated due to their permeability to both Na^+ and K^+ (Demidchik and Tester 2002) and also because they are a main pathway for Na^+ entry into the root under saline conditions (Demidchik 2007). Second, the produced ROS because of salinity stress might activate these channels and result in a massive K^+ leakage from the plant roots (Demidchik et al. 2003; Demidchik 2007; Demidchik and Maathuis 2007)

These counter mechanisms adopted by plants eventually lead to restore the cellular homeostasis leading to detoxification making survival possible under stress. The osmotic and ion stress posed by the salinity affects plant physiology at both whole plant and cellular levels (Hasegawa et al. 2000, Murphy et al. 2003).

2.6.3 Sensitivity of barley to combined stress

Barley is known as one of the most salt tolerant crops (Greenway and Munns 1980; Maas 1986; USDA-ARS 2005); but even barley is highly stressed when rising water tables carry salts to the surface (Colmer et al. 2006). High pH and sodicity are also expected in the areas affected by primary salinity (Rengasamy 2002). When it comes to secondary salinity, waterlogging plays a key role, and it increases the effect of salinity on barley (John et al. 1977). It is not just the interaction of salinity and waterlogging which affects barley, this crop is also sensitive to the single stress of waterlogging (Garthwaite et al. 2003).

2.7 Physiological and molecular mechanisms mediating plant adaptive responses to combined stress

2.7.1 Energy balance and membrane potential maintenance

Respiration is decreased when roots are not provided with efficient oxygen (like the species with less aerenchyma) under waterlogging conditions. Reduced respiration leads to energy deficiency that hinders ion transport across cell membranes, and results in a 'breakdown' of Na^+ 'exclusion' in the root of crops like wheat (Barrett-Lennard et al. 1999) and corn (Drew and Läuchli 1985). Plants under combined stresses of salinity and waterlogging will suffer greater and sooner from ion toxicity because of the increasing delivery of Na^+ (and also Cl^-) to the shoots, with decreasing K^+ uptake (Barrett-Lennard 2003). The problem gets even worse when shoot growth decreases by combined stresses and leads to reduction in incoming ions 'dilution'. It is hypothesized that root aeration traits that

help the plants to tolerate the anaerobic conditions should happen prior to conferring the combined stresses (Colmer et al. 2006). Aerenchyma, with the help of internal aeration would enable cellular respiration to continue. Continuing respiration provides the efficient energy for maintenance of membrane potential and essential transport processes for Na^+ 'exclusion' and sustained nutritional uptake like K^+ (Colmer et al. 2006). The other highly crucial electrogenic element to sustain membrane potential is the H^+ pump, therefore it is essential to keep GORK (a major selective outward-rectifying K^+ channel in root) channels closed as these channels are activated by depolarization. Membrane depolarization (by 60–80 mV) occurs considerably during salinity by itself (Shabala and Cuin 2008). Roots aerobic respiration would be restricted under waterlogging conditions and deficiency of oxygen for the roots, and it intensely leads to less ATP production. When mitochondrial oxidative phosphorylation produces 30 to 36 mol ATP, glycolysis per hexose will produce 2 to 4 mol ATP (Bailey-Serres and Voesenek 2008). It is demonstrated that ATP content drops 2-3 fold in roots under waterlogging stress (Zeng et al. 2013). Aforementioned, plant's capability to fuel H^+-ATPases, mainly for Na^+ exclusion and K^+ retention is compromised by ATP content reduction. Generally, plant metabolism is affected by unfavourable $Na^{+/}K^+$ ratios in plants, leading to severe upsurges in chlorotic and necrotic leaves and sap osmolality, significant decrease chlorophyll content, photochemical efficiency of PSII and eventually root and shoot growth (Zeng et al. 2013).

2.7.2 Cytosolic K^+ homeostasis

Plants shoot and root under combined stresses of salinity and waterlogging had more intense increase in Na^+ content and decrease in K^+ content compared to plants affected by salinity (Zeng et al. 2013). Intracellular K^+/Na^+ homeostasis is introduced as a key factor of plant tolerance to salinity (Maathuis and Amtmann (1999) , Shabala and Cuin (2008)). Intracellular K^+/Na^+ homeostasis includes two main players; first, plasma membrane SOS1 $Na^{+/}H^+$ antiporters that strongly discharges Na^+ from the cytosol (Shi et al. 2002); second, depolarization-activated outward-rectifying channels that are responsible for K^+ retention in the cytosol (Shabala and Cuin (2008), Ache et al. (2000)). Both of the transporters are dependent on O_2 availability to operate. Undeniably, SOS1 functioning relies on the presence of steep H^+ gradients across the plasma membrane which is provided by plasma membrane H^{+-}ATPase activity (Palmgren and Harper 1999).

2.7.3 ROS signalling and homeostasis

There is often an imbalance between ROS generation and scavenging under stress conditions, therefore oxidative stress is a mutual part of all "generic" abiotic stresses (Shabala et al. 2015). Increased ROS production under a wide range of abiotic stresses such as salinity and waterlogging has been reported and explained in sections 2.2.3 and 2.5.7. ROS is produced in several intracellular compartments such as chloroplasts, mitochondria, peroxisomes and also apoplast (Miller et al. 2010; Bose et al. 2014; Shabala et al. 2015). Apoplastic ROS is mediated by NADPH oxidase which is a PM-bound enzyme from the Nox family that faces the extracellular space. NADPH oxidase can be activated by salinity (Ma et al. 2011; Kaye et al. 2011) and waterlogging (Sairam et al. 2011; Baxter-Burrell et al. 2002) both at transcriptional and functional levels to produce extracellular superoxide anion, $O_2^{\bullet-}$ which is then converted to H_2O_2.

2.8 Unanswered questions and aims of this study

As shown above, salinity and waterlogging stresses on their own result in a significant disturbance to cell metabolism and crop growth and yield. The effect is much more severe when the stresses are combined. However, the physiological and molecular mechanisms behind plant adaptive responses to combined stress remain elusive. Are the detrimental effects of two stresses additive or synergistic? Which component makes the biggest contribution to plant performance under combined stress conditions? What physiological mechanisms (and, ultimately, what genes) are most essential to combined stress tolerance? How can these be targeted in breeding programs? To answer these questions, three set of experiments including whole plant study under soil and hydroponic conditions in glasshouse followed by specific ions exchange studies with MIFE were designed and carried out in this work.

Chapter 3: Materials and Methods

3.1 Glasshouse Soil Experiment

3.1.1 Plant Material

Twelve varieties of barley (*Hordeun Vulgare* L.) contrasting in their tolerance to salinity were selected for the present study. All the seeds were provided by the Australian Winter Cereal Collection and multiplied at the Launceston facilities of the Tasmanian Institute of Agriculture (TIA). A list of the varieties, their salinity tolerance and their origin is given in Table 3.1. The salinity tolerance of selected barley varieties based on their damage index is shown in Figure 3.1 (Wu et al. 2014).

Table 3.1. Selected barley varieties, their origin and tolerance to salinity stress

Variety	Origin	Tolerance to Salinity
Naso Nijo	Japan	Sensitive
ZUG 403	China	Sensitive
Gairdner	Australia	Sensitive
YSM1	China	Moderately Tolerant
Franklin	Australia	Moderately Tolerant
Mundah	Australia	Moderately Tolerant
Yerong	Australia	Moderately Tolerant
YU6472	China	Moderately Tolerant
ZUG 293	Sudan	Tolerant
Gebeina	China	Tolerant
YYXT	China	Tolerant
CM72	USA	Tolerant

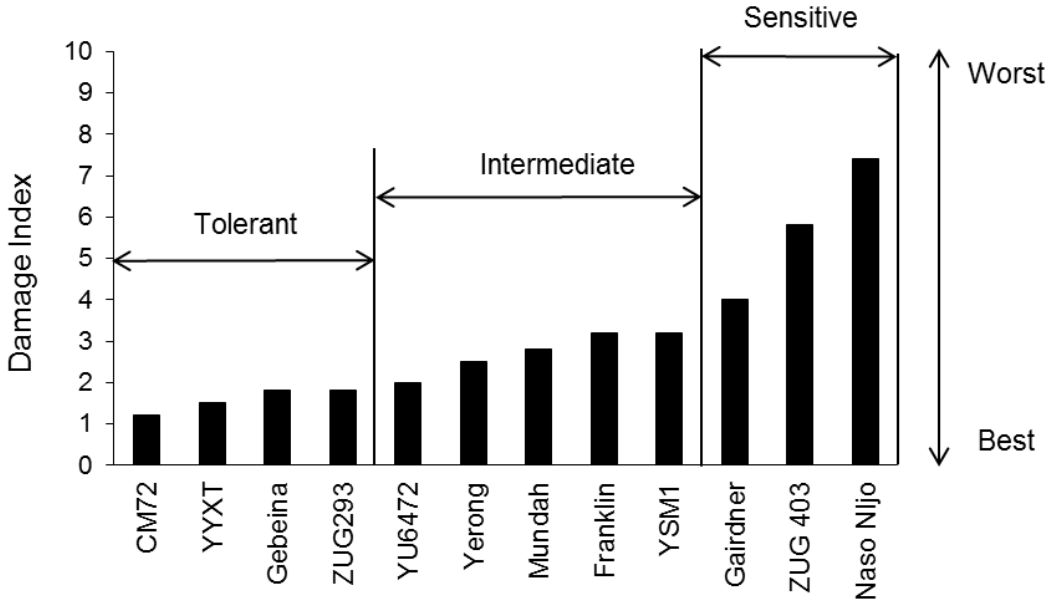

Figure 3.1. Modified figure of selected barley varieties tolerance to salinity based on their damage index (Wu et al. 2014)

3.1.2 Experimental design

The experiment was set up in a randomised complete block design with 12 barley varieties. Four treatments, 250mM NaCl (NaCl), waterlogging (WL) and combined waterlogging and salinity (WL/NaCl) were applied with 6 replicates (6 plants per pot, 4 pots each treatment). Control and NaCl-treated plants were randomly arranged on the benches in the glasshouse of the Tasmanian Institute of Agriculture (TIA), Hobart, Tasmania. Pots were placed in rectangular containers (70 x 40 x 30 cm) filled with water to apply both non-saline and saline waterlogging conditions.

Figure 3.2. Barley plant seeds were planted in 6-inch pots (1.5 L) with potting mix, 6 seeds per pot.

3.1.3 Growth conditions

Barley seeds were planted in 6-inch pots (1.5 litre capacity) filled with potting mix, 6 seeds per pot. The bulk density of the potting mix was ~0.5 kg L^{-1}, and the composition (by volume) was: 80% composted pine bark, 10% sand and 10% coir peat, plus fertiliser (N:P:K ratio equal to 8 : 4 : 10) at 1 kg m^{-3}; dolomite; 8 kg m^{-3}; wetting agent 0.75 kg m^{-3}; sulfate of iron 1 kg m^{-3}; gypsum 1 kg m^{-3}; isobutylenediurea (IBDU) 1 kg m^{-3}; trace element mix 0.75 kg m^{-3}; zeolite 0.75 kg m^{-3}; and pH 6.0. A saucer was placed under each pot to retain salt and other nutrients. Plants were hand-watered with tap water for twice a day for 8 days. Settings for the glasshouse were: 25/18 (±1) C day/night temperature and 65/80 (±5) % day/night relative humidity, with natural sunlight levels and variable photoperiod depending on the time of year. On the 8th day, the treatments were applied to the plants as described below.

3.1.4 Treatments

Barley seedlings were assigned to four different treatments including each 250mM NaCl and waterlogging and their combination to compare with the respective control plants. The treatments are explained in Table 3.2.

Table 3.2. Treatments applied to the barley seedlings

Treatment	Description
Control	Irrigation with tap water, No NaCl, Well drained
NaCl	Irrigation with 250mM NaCl solution, well drained
WL	Pots with seedlings were placed in containers filled with tap water and submerged under water by 1 cm
WL/NaCl	Pots with seedlings were placed in containers filled with 250mM NaCl solution and submerged by 1 cm

3.1.5 Sampling

Measurements were conducted in two steps: after 10 and 15 days of stress. First measurements were conducted on the 10^{th} day including chlorophyll content measurements using SPAD meter (SPAD-502, MINOLTA, Japan) with the measurements from the first leaf (oldest leaf) on each plant. The early chlorophyll content measurements on day 10 were driven by the fact that sensitive varieties showed strong signs of necrosis and chlorosis of the first leaf by day 15 (when all other measurements were conducted). By 15^{th} day, four nominated varieties that showed higher SPAD value on day 10, were measured for chlorophyll content again. Four other parameters measured on the day 15 of the treatments included: chlorophyll fluorescence measurements, fresh (FW) and dry weight (DW) estimation and Na^+/K^+ content.

3.1.6 Measurements

Measurements were divided in two groups: non-destructive and destructive. Specific details are given below.

3.1.6.1 Non-Destructive Measurements

Non-destructive measurements included:

- Chlorophyll Fluorescence
- Chlorophyll Content

➢ **Chlorophyll Fluorescence**

Chlorophyll fluorescence measurements help to understand the photosynthesis itself as well as provide information on the mechanisms of different environmental and anthropogenic stresses that affect the photosynthetic capacity of the plant (Bolhar-Nordenkampf et al. 1989). Chlorophyll fluorescence was measured by Chlorophyll Fluorometer OS-30p (Opti-Sciences, USA). Due to its non-destructive and non-invasive nature, this device can be used during the plants growth.

Figure 3.3. Chlorophyll Fluorescence meter. Inserts show: clips used during the measurements to keep the plant leaf and the monitor presenting measured parameters such as F_o, F_m and F_v/F_m

The basis of chlorophyll fluorescence measurements

The light absorbed to the leaves by the chlorophyll molecules can be driven to three different paths. It can be either used for photosynthesis which is the main aim of light absorption, or it can be lost as heat or re-emitted as fluorescence. When the photosynthesis efficiency decreases, the efficiency of the other two paths, heat and fluorescence, increases; therefore we can assume the photosynthesis efficiency by measuring the fluorescence of the leaves (Maxwell and Johnson 2000). Therefore, chlorophyll-fluorescence analysis is an indicator of plant health and is used to estimate PSII activity during stress which varies with plant water status. Therefore, it is one of widely used parameters for measuring leaf physiological status. Dark adapted measurement of Fv/Fm (ratio of variable to maximal fluorescence) reflects maximum quantum efficiency of PSII photochemistry (Baker 2008). Decrease in Fv/Fm indicates extent of increased leaf damage.

Chlorophyll Fluorescence analysis

The theory of inverse relationship between photosynthesis and chlorophyll fluorescence first came from Kautsky and Hirsch (Kautsky and Hirsch 1931). The graph below is called Kautsky Effect after the scientist ((Krause and Weis 1984) Figure 3.4). Kautsky effect has two phases: a sharp rise from the bottom line of fluorescence (F_o) to the maximum level of fluorescence ($F_{m)}$ and then a very slow decline to the steady state of F_s (Lichtenthaler and Miehe 1997).

Three parameters of Chlorophyll fluorescence were measured in the current study including F_m, F_o and F_v/F_m. F_o is the ground Fluorescence, F_m is the maximum fluorescence and F_v is the variable fluorescence estimated as the difference between F_m and F_o: $F_v= (F_m-F_o)$. Table 3.3 clarifies the measured Fluorescence characteristics in this experiment and their physiological explanation (DaMatta and Hemantaranjan 2003).

Table 3.3. Basic Fluorescence Characteristics and their Physiological Meaning

Symbol	Name	Physiological Meaning	Estimation
Fo	Minimal Fluorescence of Dark Adapted Sample	Effectiveness of coupling between antennae chlorophyll and reaction centres of PSII	Measured directly
Fm	Maximal Fluorescence of Dark adapted Sample	Maximal capacity of PSII to transfer electrons from reaction centre through ETC to PSI	Measured directly
Fv	Variable Fluorescence	Amount of efficiently working PSII units	Fm-Fo
Fv/Fm	Maximum quantum yield of PSII	Proportion of efficiently working PSII units among the total PSII population	(Fm-Fo) / Fm

Six replicates were assigned for chlorophyll fluorescence measurements per treatment. Plants were transferred to the laboratory and dark adapted for 20 minutes before the measurements. Top one third of the first leaf (oldest leaf) was used in the measurement. A clip was used to hold the leaf to ease locating the device on the spot. The clip also provided standardising the site measured on all the leaf samples.

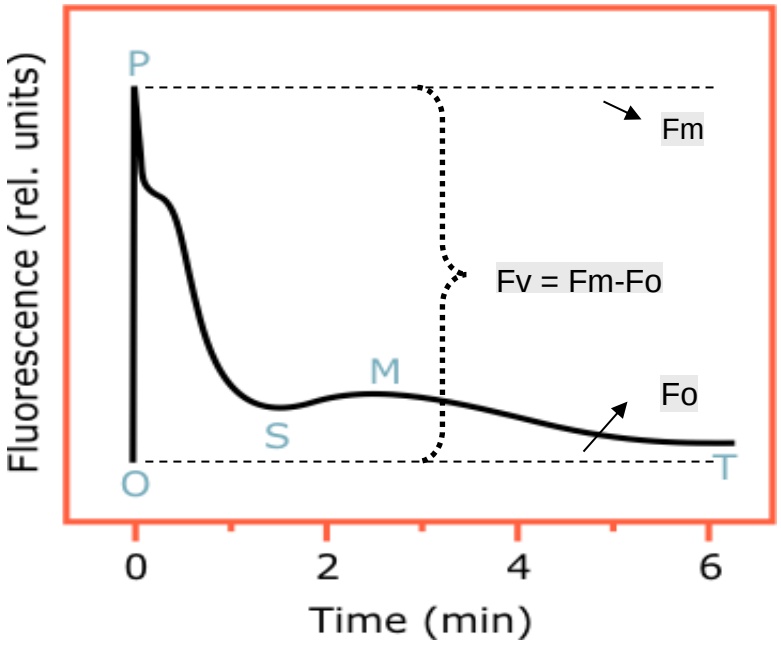

Figure 3.4. Kautsky effect Graph

- **Chlorophyll Content**

Chlorophyll content is another non-destructive method that provides data about the physiological status of the plant (Gitelson et al. 2003). Chlorophyll content shows the photosynthetic activity of nitrogen concentration in the process of growth in plants and also measures the crop reaction to nitrogen application (Haboudane et al. 2002). The device measures the greenness of leaves as reflected by the chlorophyll content and N status. It provides information about changes in the plant well before the visible symptoms appear.

The first one third of the tip of the oldest leaf was used for the measurements using SPAD meter (SPAD-502, MINOLTA, Japan). Six replicates were assigned for each treatment. The chlorophyll content was measured during the day light in the glasshouse using intact plants in the pots.

Figure 3.5. Chlorophyll content, SPAD meter (SPAD-502, MINOLTA, Japan)

3.1.6.2 Destructive Measurements

Destructive methods used included:

- Fresh (FW) and dry (DW) weight estimation
- Na^+ and K^+ contents

➢ **Fresh and dry weight estimation**

Plants were harvested after 15 days of treatment (23 days old plants). Four replicates per variety per treatment were used. The shoots were cut gently just above the soil surface and fresh weight was estimated by weighting the plants immediately, using analytical scales (A & D Weighing Scale, d=0.1mg, Japan). Cut shoots were placed in absorbent paper bags and dried at 65°C for 3 days in a dryer followed by DW weighing using the same analytical scales.

- **Na^+ and K^+ contents**

Plant dry material was required to be acid digested by microwave digestion prior measuring Na^+ and K^+ content by flame photometer.

Microwave Digestion:

The dried plant shoots were digested using a microwave Reaction System Mars6 (Figure 3.6 a) prior to measuring ion content. Approximately, 0.1 g of dry matter of shoot samples was weighed from different parts of the shoot including stems and leaves, representing the whole shoot. The plant material was placed in the EasyPrep vessels for digestion (Figure 3.6 d). All the work was conducted in a fume hood. Seven ml of Nitric Acid were added to the vessels. The vessel was covered with the lid and the control covered with an integrated thermowell for all TFM wetted surfaces and then tightened in the vessel holder (Figure 3.6 d). Each round of the digestion process included 11 samples plus a control processed simultaneously (Figure 3.6 c). Theontrol vessel had 7ml of nitric acid without any plant material. The fibre optic temperature probe was placed in the standard vessel to provide accurate temperature measurements of the vessels (Figure 3.6 b). Unlike metal thermocouples, which can self-heat in the microwave and give inaccurate readings, CEM's fibre optic temperature probe provides precise measurement every time. The machine warms up the vessels for 15 minutes to reach 200°C. The temperature remains at 200°c for 10 minutes and cools down for 15 minutes. When the machine turns off, the solution inside it has the temperature around 80°C and remains in the fume hood for a further hour to cool completely before it can be opened. The chambers have to be opened in the fume hood due to the acid vapours escaping the vessels once they are opened. The digested plant material, in a solution of acid is transferred to 15ml plastic tubes and doubled distilled water is added to achieve a final volume of 15ml.

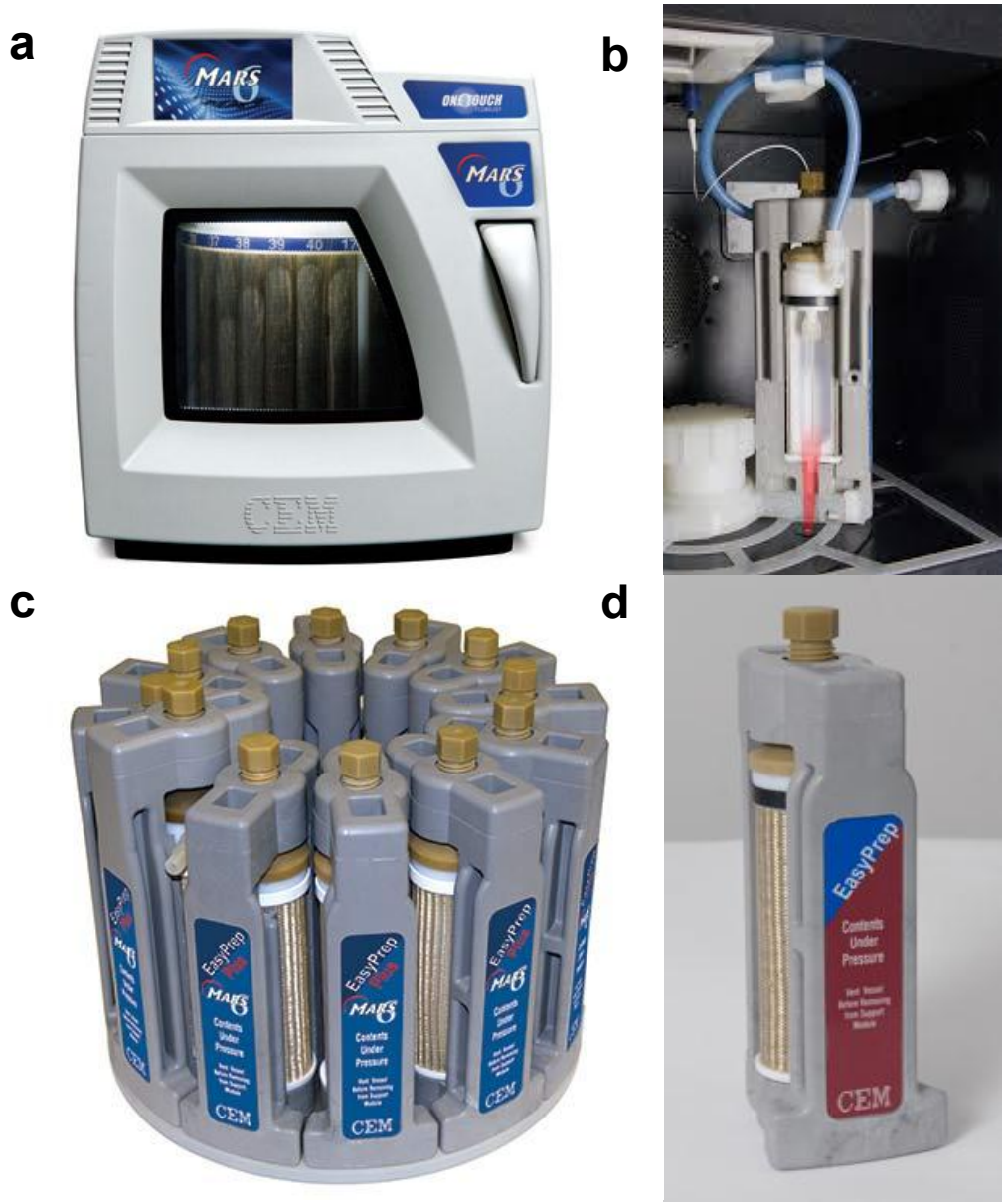

Figure 3.6. a. Microwave Reaction System Mars6; b. The standard vessel to control the temperature and pressure inside of the vessels; c. The vessels tray; d. the sample vessel and the holder

Flame Photometer:

Sodium and potassium contents in the leaves were determined by flame atomic emission spectrometry using specific filters (Hald 1947).The samples were diluted to bring the amounts of Na^+ and K^+ below 50 and 10 ppm, respectively. In the flame-photometer, the solution was sprayed through an atomizer into a chamber, where it was extracted into a flame. Intensity of light emitted from the flame is proportional to the concentration of the specific ion in the solution. Combustion of the elements produced a light of a particular wavelength (λ max for Na = 589 nm and for K = 766 nm). The light generated was conducted

through the selected filters to impinge upon a photoelectric cell which set off a galvanometer, causing the digital reading of the respective samples. Na^+ and K^+ concentrations were determined based on standard curves of known concentrations.

3.1.7 Data analysis

The data collected from all measured parameters were analysed by IBM SPSS statistics 20 (IBM, New York, USA). Univariate General Linear Model with the Duncan test was used to confirm the significant difference between treatments and varieties. Bivariate correlation was used to determine the significant correlation between the characteristics that has been measured (biomass, chlorophyll content, chlorophyll fluorescence and Na^+/K^+ content).

3.2 Glasshouse Hydroponic Experiment

3.2.1 Plant Material

Eight barley varieties (*Hordeun Vulgare* L.) contrasting in their tolerance to salinity were selected. The seeds were obtained from the Australian Winter Cereal Collection and multiplied in Launceston using TIA facilities. Table 3.4 presents the varieties, their origin and their tolerance to salinity. Furthermore, the tolerance of selected barley varieties based on their damage index is compared in Figure 3.7 (Wu et al. 2014).

Table 3.4. Selected barley varieties, their origin and tolerance to salinity stress

Variety	Origin	Tolerance to Salinity
YU6472	China	Moderately Tolerant
TX9425	China	Tolerant
YYXT	China	Tolerant
YSM1	China	Moderately Tolerant
Franklin	Australia	Moderately Tolerant
Mundah	Australia	Moderately Tolerant
Gairdner	Australia	Sensitive
Naso NIjo	Japan	Sensitive

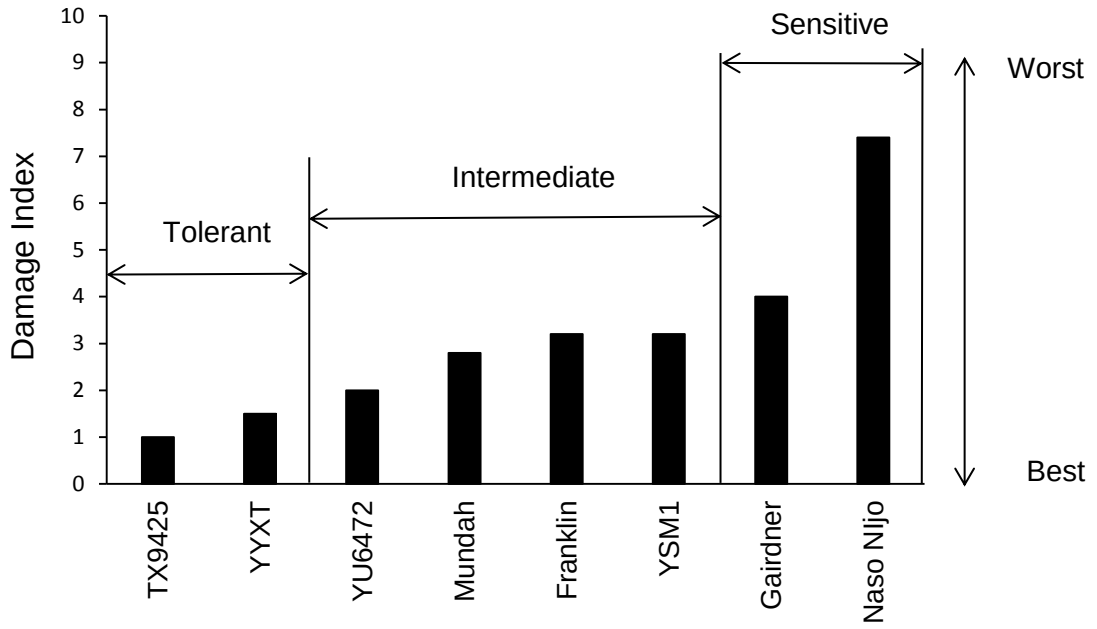

Figure 3.7. Modified figure of selected barley varieties salinity tolerance based on their damage index (Wu et al. 2014)

3.2.2 Experimental Design

The experiment in the glasshouse was set up in a randomized complete block design with 8 varieties of barley. Four treatments including control, 150mM NaCl (NaCl), waterlogging (WL) and their combination (WL/NaCl) as described below were applied with 8 replicates. Each treatment was planted in a separate designed hydroponic container which was randomly located in the glasshouse of the Tasmanian Institute of Agriculture (TIA), Hobart, Tasmania. All containers were supplied with aeration system to provide the oxygen for the roots in the solution before applying the treatments and also after treating NaCl stressed plants and in control experiments.

3.2.3 Growth Condition

A solution culture experiment was conducted to study the responses of 8 barley varieties of varying salinity tolerance. In order to reduce the risk of low germination in some varieties and improve the efficiency of the limited space in hydroponic experiments, seeds were grown under the same conditions in the growth room. Afterwards healthy seedlings were transferred to the main space in the glasshouse to apply the treatments. Seeds were surface sterilized with 0.5% v/v sodium hypochlorite (commercial bleach) for 10 minutes followed by a thorough

rinsing under the tap water for 30 minutes to ensure the absence of bleach residue on the seeds. Once the rinsing was completed, seeds were placed in cavities of a punched plate sited on the top of a 500 ml container containing double distilled water. Plants were grown in the containers for 4 days under light-temperature controlled conditions with a 16/8 light/dark cycle and 24°C/ 20°C day/night temperature and 65/80 (±5) % day/night relative humidity. The solution inside each container was aerated by an air stone connected to an air pump (Fig. 3.8).

Figure 3.8. Barley seeds were grown in 500 ml containers containing double distilled water with aeration. Seedlings were grown for four days in light-temperature controlled environment prior to relocation to a glasshouse.

The seedlings were transferred to 5 litre containers to the glasshouse on the fourth day. Settings for the glasshouse were: 25/18 (±1) C day/night temperature and 65/80 (±5) % day/night relative humidity, with natural sunlight levels and variable photoperiod depending on the time of year. Eight holes 2cm in diameter were drilled in a lead of a 5L container and a sponge holding a seedling was inserted in each hole (Fig. 3.9). The containers were filled with half strength Hoagland solution. The composition of the solution used was: 2.5 ml/L KNO_3, 2.5 ml/L $Ca(NO_3)_2$, 1 ml/L $MgSO^4$, 0.5 ml/L Micro elements (including H_3BO_3, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $H_3MoO_4 \cdot H_2O$, $Na_2MoO_4 \cdot 2H_2O$), 0.5 ml/L Fe EDTA, 0.5 ml/L KH_2PO_4. The seedlings were assigned to four mentioned treatments on the sixth day of planting, the second day after transferring to the main containers. The treatments were not applied gradually due to the nature of combined waterlogging and salinity that occurs suddenly and in a short term. The containers were arranged in a randomised complete block design with four replicates. Control plants had half Hoagland strength solution. Plants

were exposed to salt stress by replacing the container solutions with a half Hoagland strength solution containing 150mM NaCl. Hypoxic conditions in waterlogging experiments (either WL only or WL/NaCl) were provided by applying 0.2% agar to the solution as detailed below. Both single (WL) and combined (WL/NaCl) waterlogged solutions were bubbled with Nitrogen gas for an hour to ensure the absence of oxygen in the solution. The solutions were changed every three days to ensure relatively constant ion composition and pH was maintained around pH 6.0-6.5. The treatments were maintained for 16 days. After 8 and 16 days stress, first and second sampling was conducted, respectively.

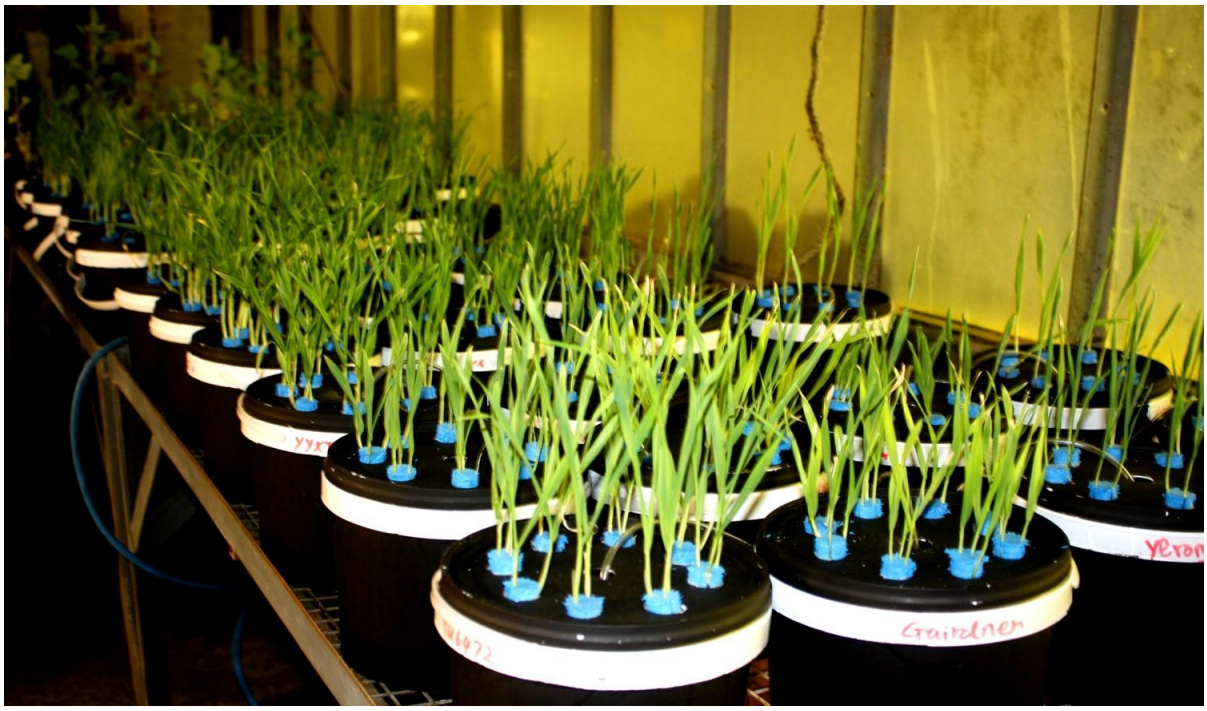

Figure 3.9. Barley plants were grown in hydroponic solution in light-temperature controlled glasshouse.

3.2.4 Treatments

Four treatments were assigned to record the responses of barley varieties to NaCl, WL and the combination to compare with control plants. All plants were grown for 4 days in a growth room and for 2 days in a glasshouse in a half strength Hoagland solution and treatments were applied from day 6 after planting.

Control plants received the half strength Hoagland solution for the duration of the experiment (22 days in total). NaCl stress was applied by adding 150mM NaCl to the half strength Hoagland solution and the solution was bubbled 24/7 with air pump to ensure enough oxygen around the roots. To minimize the oxygen uptake from the roots in plants

under WL and WL/NaCl stress, agar solution was prepared. 0.2% agar was added to a half strength Hoagland solution, heated to boiling point in a microwave and was stirred on a magnetic stirrer (for smaller amount) or by a commercial stirrer (for bigger containers) over night to cool the solution gradually to avoid any clusters of agar in the solution. The agar solution was poured gently to the main container to avoid entering any air bubble while pouring and then the seedlings were inserted into the sponge and into the holes in the lid. The half strength Hoagland agar solution was then bubbled with a nitrogen gas for an hour to ensure absence of oxygen. A similar procedure was used in a combined waterlogging-salinity stress. The only difference was that 150mM NaCl was added to the half strength Hoagland solution while agar and N_2 gas were applied in a similar manner.

3.2.5 Sampling

Two sets of sampling were scheduled to compare the responses of the plants during 8 and 16 days. In each sampling round, two groups of samples were collected. The first group was used for measuring biomass. Four replicates per plant per treatment were collected and transferred to the laboratory for measuring the fresh weight and length, afterwards they were dried and dry weight was estimated. For the second group plant material was collected in Eppendorf tubes and frozen. These samples were squeezed to extract the sap for measuring osmolality and Na^+/K^+ content.

3.2.6 Measurements

3.2.6.1 Non-Destructive Measurements

Non-destructive measurements included:

- Chlorophyll Content
- Chlorophyll Fluorescence

Non-destructive measurements are described in chapter 3.1.6 in details.

3.2.6.2 Destructive Measurement

Destructive methods used included:

- Fresh and Dry weight estimation
- Osmolality
- Na and K Contents

Details are given in chapter 3.1.6.

- **Fresh and Dry Weight**

Barley plants were harvested for biomass measurements after 8 and 16 days of stress. Four replicates per variety per treatment were used. Plants were carefully removed from the sponge holding the plants in the container's lid holes. The roots were gently rinsed with running water to wash the salt and agar solution off the roots. The roots were separated from the shoots and placed in a wet towel to prevent drying during transfer to the laboratory. Both shoots and roots were weighed separately using analytical scale (A & D Weighing Scale, d=0.1mg, Japan). Plant material was placed in absorbent paper bags and dried at 65°c in dryer for 3 days to constant weight to determine dry weight.

- **Osmolality**

Four replicates per variety per plant were harvested. Barley plants after 8 and 16 days of stress and control plants were gently taken out of the sponge holders and roots were carefully rinsed under running water to wash off the salt and agar solutions. Roots and shoots were placed in Eppendorf tubes separately and frozen overnight. For sap extraction, the frozen shoots and roots were thawed and squeezed in the respective Eppendorf tube using a stick. The extracted sap was centrifuged at 3600 rpm speed for 15 minutes using a centrifuge (Eppendorf Centrifuge 5804 R). Centrifuged sap was analysed using an osmometer (Wescor, Vapro Pressure Osmometer 5520).

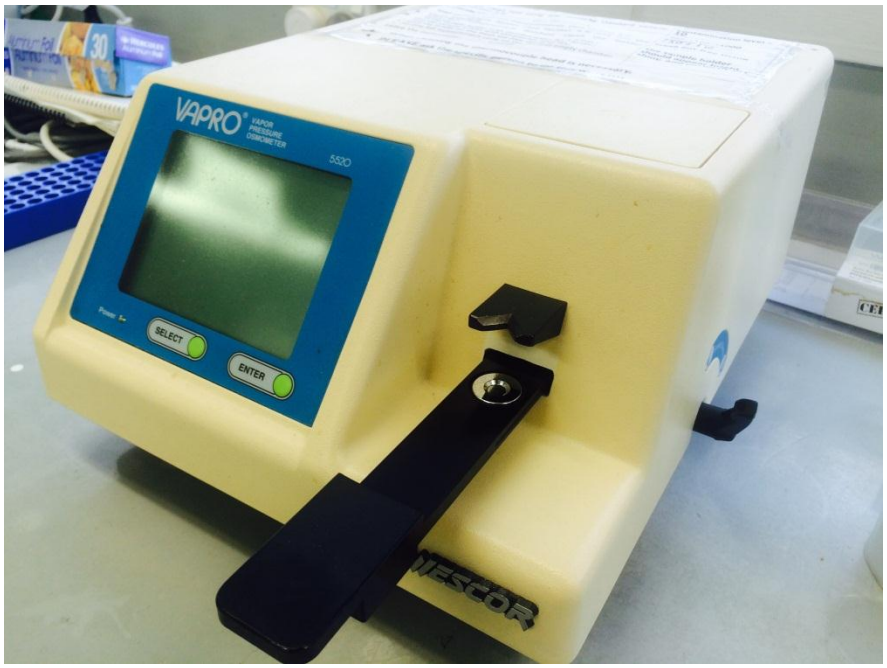

Figure 3.10. Osmometer (Wescor, Vapro Pressure Osmometer 5520)

- **Na^+ and K^+ Contents**

Dry material digestion and Na^+/K^+ content measurements are discussed in details in 3.1.6.2b.

3.2.7 Data Analysis

Data analysis is discussed in details in 3.1.7

3.3 MIFE

3.3.1 Plant material

Three barley varieties were chosen from previous whole plant study experiments under soil and hydroponic conditions in a glasshouse, representing high, medium and low tolerance to combined WL/NaCl. Barley seeds were obtained from Australian Winter Cereal Collection and multiplied using the TIA facilities in Launceston. A list of varieties illustrating their origin and their tolerance to the combined WL/NaCl is shown in Table 3.5.

Table 3.5. Selected barley varieties, their origin and tolerance to combined WL/NaCl stress

Variety	Origin	Tolerance to WL/NaCl
ZUG403	China	Sensitive
Gebeina	China	Tolerant
YU6472	China	Moderately Tolerant

3.3.2 Growth conditions

Seeds were surface sterilized with 0.5% v/v sodium hypochlorite (commercial bleach) for 10 minutes followed by a 30 minutes thorough rinsing under tap water to ensure the absence of residual bleach on the seeds. Washed seeds were then placed in growth room as described earlier in section 3.2.3 for three days. Three day old seedlings were exposed to four explained in Table 3.6 for 2 days (Figure 3.1). More details are given in Table 3.6.

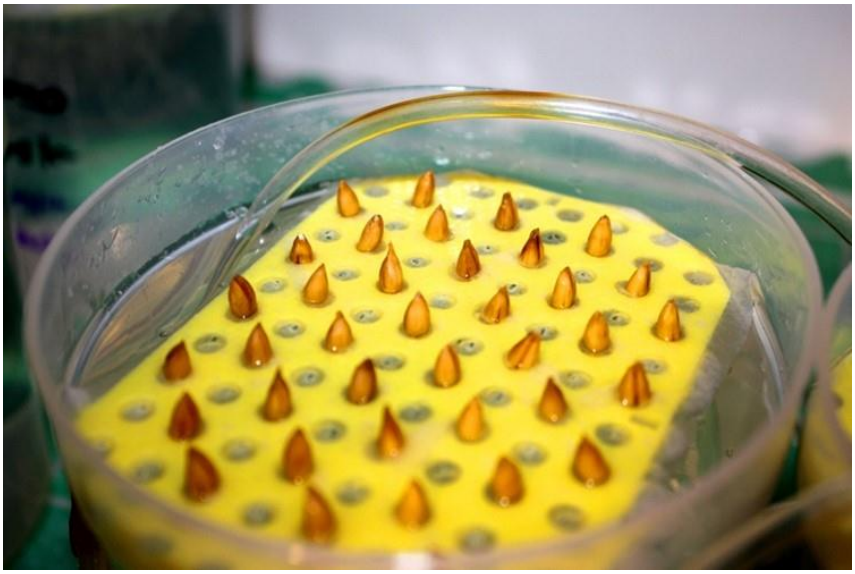

Figure 3.11. The seeds were planted in punched plastic plates on the top of 500 ml container filled with double distilled water

Table 3.6. Preparation and treatment of barley seedlings in growth room

Treatment	Description
Control	Distilled water in containers was replaced with basic nutrition solution (BSM) containing 0.5 mM KCl and 0.1 mM CaCl2, pH 5.5, aerated using air stones (Freshwater aquarium air stones)
NaCl	Distilled water in containers was replaced with BSM solution containing 150 mM Nacl, pH 5.5, aerated using air stones (Freshwater aquarium air stones)
WL	Seedlings were transferred to a specific MIFE chambers with the seminal root being separated from others and immobilized in the chamber making it ready for MIFE measurements. A set of 6 chambers were placed in a container filled with 0.2% agar prepared in the BSM solution. The solution was bubbled with nitrogen gas for 20 minutes to ensure the absence of oxygen.
WL/NaCl	Condition and preparation were the same as in WL treatment, the only difference was that the BSM/agar solution also contained 150mM NaCl.

Treatments chambers with seeds were kept in darkness for 2 days before the measurements were taken.

3.3.3 MIFE theory

The movement of chemicals in solution is influenced by flow of chemical forces and it is directed towards lower concentration areas. In the presence of an electrical field, ions are also influenced by electrical forces. These chemical and electrical driving forces and also other parameters of the solution and an ion can explain the ion movement in a solution. The net flux of an ion is typically measured in units of nmol m^{-2} s^{-1}. The net flux ion is estimated by measuring voltage changes of an ion selective microelectrode when it is moved between two positions: close to and further away from the object of study. Therefore, the MIFE technique is a non-invasive method with a relatively high temporal (resolution of 10 seconds) and special (20 micrometer) resolution. A measuring chamber is mounted on a microscope thus enabling monitoring microelectrode movement above the tissue in study.

At first, the microelectrode's tip that is filled with the liquid ion exchanger (LIX) stays within distance x from the tissue into which ions are moving with a net flux J. The absence of bulk solution flow is assumed, and therefore, ionic movement in the solution (regardless of the membrane transport processes) is only by diffusion under the electric influence and chemical forces in solution. It is also expected that the measurement is close to the surface and that the ionic movement is normal to the surface. μ (joules mol^{-1}) is defined as the electrochemical potential in the solution at the distance x. Due to the LIX permitting free ion passage in question (but not others), the electrochemical potential of the ion inside the electrode is also μ. The μ chemical component inside the electrode is fixed by the concentration of the filling solution. Where V (volts) is measured by an electrometer connected through suitable half cells to the electrode solution and to a reference electrode some distance away in the bath solution, z is ion's valence and F is the Faraday number, zFV is the electrical component.

Afterwards, the microelectrode gently and slowly moves away from the tissue to the distance dx. The electrochemical potential in this second position is $\mu + d\mu$ which is the same as inside the electrode, even though the electrical component has changed and measured voltage is now $V + dV$.

Based on the basic electrochemical theory (Newman, 2001), the net ionic flux *J* is given in terms of the ion concentration **c** (mol m^{-3}), the mobility of the ion *u* (speed per unit force, m s^{-1} per newton mol^{-1}), and the force per mole which is the electrochemical potential gradient ($d\mu/dx$). Consequently, $J = c\ u\ (d\mu/dx)$. $d\mu$ both inside the electrode and in the solution equals to $zFdV$. Therefore, the flux may be written as:

$$J = c\ u\ z\ F\left(\frac{dV}{dx}\right) \quad (1)$$

When the electrode is calibrated in standard solutions, the concentration is known or adequately measured by the value of *V*. *u* and *z* are known constants for the ions, even though for multi-valent ions *u* depends on *z*. *dV* is measured by electrometer while the electrode moves between the chosen distance *dx*. Due to LIX imperfection, electrodes are required to be calibrated to recognize its actual "Nernst slope" (which is determined by the valence). The basic equation for ion flux measurements is:

$$J = c\ u\ F\left(\frac{58}{Nernst}slope\right)\left(\frac{dV}{dx}\right) \quad (2)$$

The theory can also be stated in terms of the diffusion coefficient $D\ (= u\ R\ T)$ for the ion instead of the mobility *u*. The geometry of the object would affect flux calculations. In the case of cylindrical geometry (e.g. a root surface), the radius of the cylinder (*r*) should be taken into account. It is applicable by replacing *dx* in the implementation of the equation above by:

$$dx = r^2\left[\frac{1}{r+x} - \frac{1}{r+x+dx}\right] \quad (3)$$

Also in the case of spherical geometry (e.g. a protoplast), *dx* should be:

$$dx = r\ ln\left[\frac{r+x+dx}{r+x}\right] \quad (4)$$

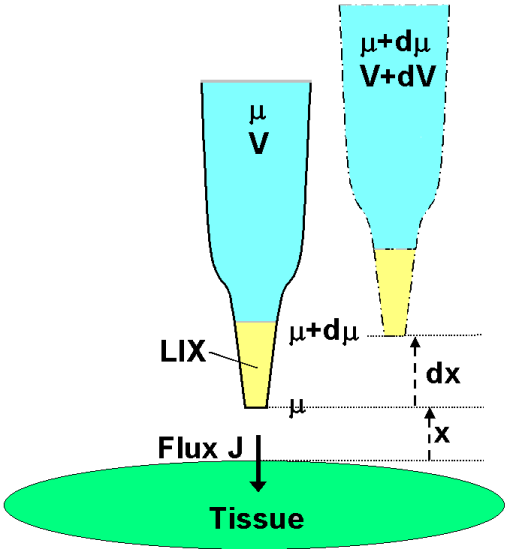

Figure 3.12. Electrode movement during MIFE measurements (Newman 2001)

3.3.4 Electrode fabrication and calibration

Electrode fabrication and calibration is the first step in MIFE measurements. MIFE microelectrodes are ion-selective liquid type electrodes. The advantage of liquid type microelectrodes is the rapid response compared to solid type ion selective microelectrodes. MIFE microelectrodes were prepared on a daily basis and calibrated at least twice a day (before and after the experiments). Electrode preparation can be split into several steps: (1) preparation of electrode blanks; (2) filling up electrodes; (3) calibrating ion selective microelectrodes.

1. Electrodes were pulled out from non-filamentous glass capillaries (Harvard Apparatus, 30-0053, GC150-10) to tip diameter ~1 μm. The pulled electrodes were dried in the oven at 225°C overnight, and salinized with tributylchlorosilane (90796, Fluka Chemicals) to make their surface hydrophobic. The prepared electrodes were then able to be stored for several weeks under the cover.

2. On the day of the measurement, an electrode blank was mounted on a microscope stage of a filling station and the electrode tip was broken against a flat glass surface to 2-3 μm in diameter. The electrode then was backfilled with the appropriate backfilling solution followed by a front filling with the respective liquid ion exchanger (LIX). Special attention was paid to the absence of air bubbles in the electrode tip and the length of LIX shaft that should not exceed 200 μm in length (Table 3.7).

Table 3.7. Specific details about the major types of commercially available LIX and backfilling solution

Ion	Catalogue No	Ionophore	Backfilling Solution (mM)
H^+	95297	4-Nonadecylpyridine	15 NaCl+40 KH_2PO_4
K^+	60031	Valinomycin	200 KCl

Prepared electrodes were placed in an electrode holder filled with BSM and left for conditioning. The H^+ selective electrodes require up to an hour for conditioning. Other ion selective electrodes used (K^+) are ready for use immediately after preparation.

Prepared ion selective electrodes were calibrated in a set of three respective standards that cover the range of the expected ion concentration (Table 3.8). The quality of prepared electrodes was assessed after the calibration. The electrodes with a slope below 50 mV/decade for monovalent ions, and 25 mV/decade for divalent ions and correlation below 0.999 were discarded from use.

Table 3.8. Calibration standards of electrodes

Measured Ion	1st Calibration Standard	2nd Calibration Standard	3rd Calibration Standard
H^+	~5.00 pH	~6.00 pH	~7.00 pH
K^+	250 µM	500 µM	1000 µM

3.3.5 Experimental Protocols

A vertical MIFE set-up configuration with a vertical measuring chamber was used in MIFE measurements to minimize the BSM surface area exposed to the air that is critical in hypoxic (waterlogging) experiments (Fig. 3.13). Plants which were under stress conditions for two days were used in MIFE experiments. A seminal intact root from the whole seedling

was immobilized in a measuring chamber with the help of silicone stoppers and the chamber was filled with the same treatment solution as detailed in Table 3.6 (Figure 3.13).

Immobilized plants were left for 30 minutes' adaptation before the experiments were resumed. Measurements were conducted from the mature zone (MZ) (indicated by arrows in Figure 3.13).

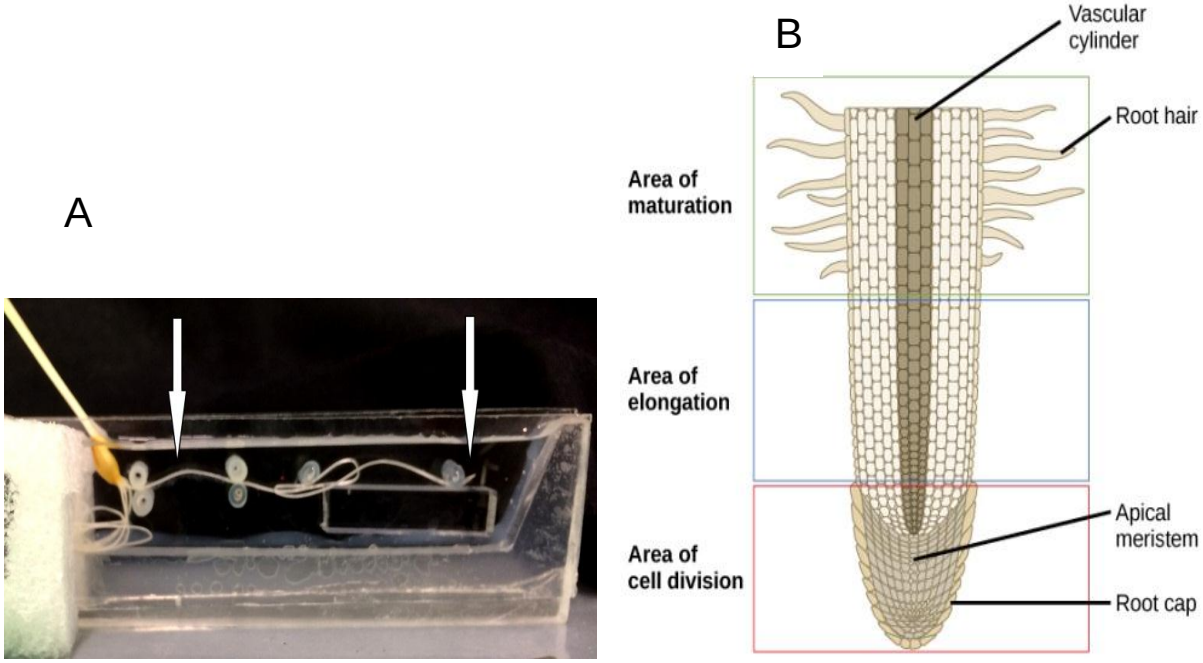

Figure 3.13. A. Barley root immobilised in the MIFE chamber. Arrows point out towards the mature and elongation zones on the root. **B.** Plant root with indication of specific root zones.

3.3.6 MIFE measurements

Prepared microelectrodes were placed in electrode holders over a microscope stage, centered with ~3 µm spacing between them and co-focused (Figure 3.14). Up to two ions were measured simultaneously and essentially from the same site on the root. A measuring chamber with the immobilized root was placed on a microscope stage and the microelectrodes were positioned ~40 µm away (position M1, Figure 3.15) from the respective zone and the measurements were resumed. Electrodes were moved ~121 µm away from the root (to position M2) and back to position M1 with 6 sec recordings at each position. The measurements were conducted for approximately 10 minutes at each site to enable steady readings, then the chamber was replaced with a new one and the measurements resumed. Six to ten plants were assessed for each treatment.

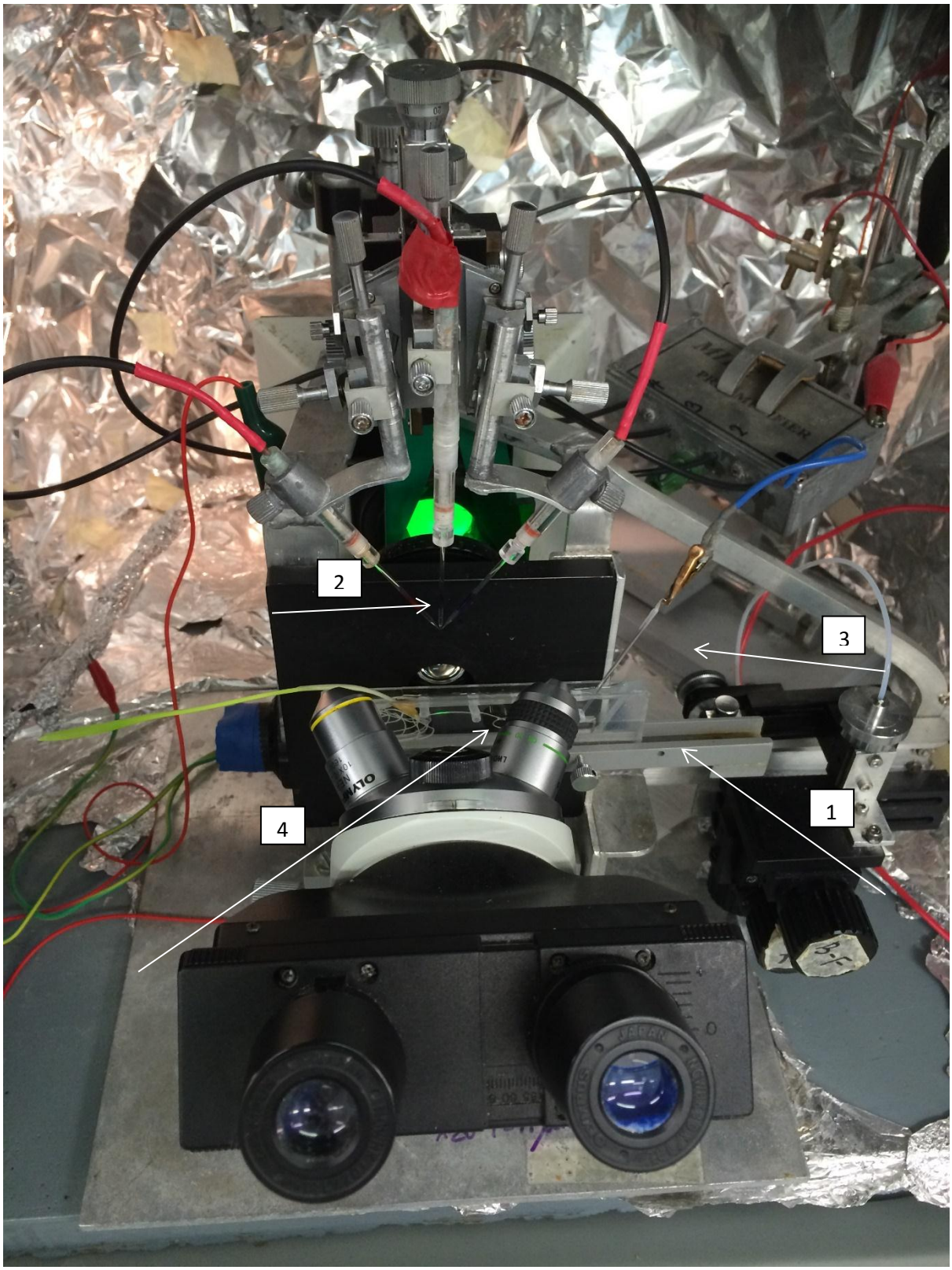

Figure 3.14. MIFE set-up, representing electrode holders (1) with microelectrodes (2), a reference electrode (3) and a chamber with immobilised intact barley root (4)

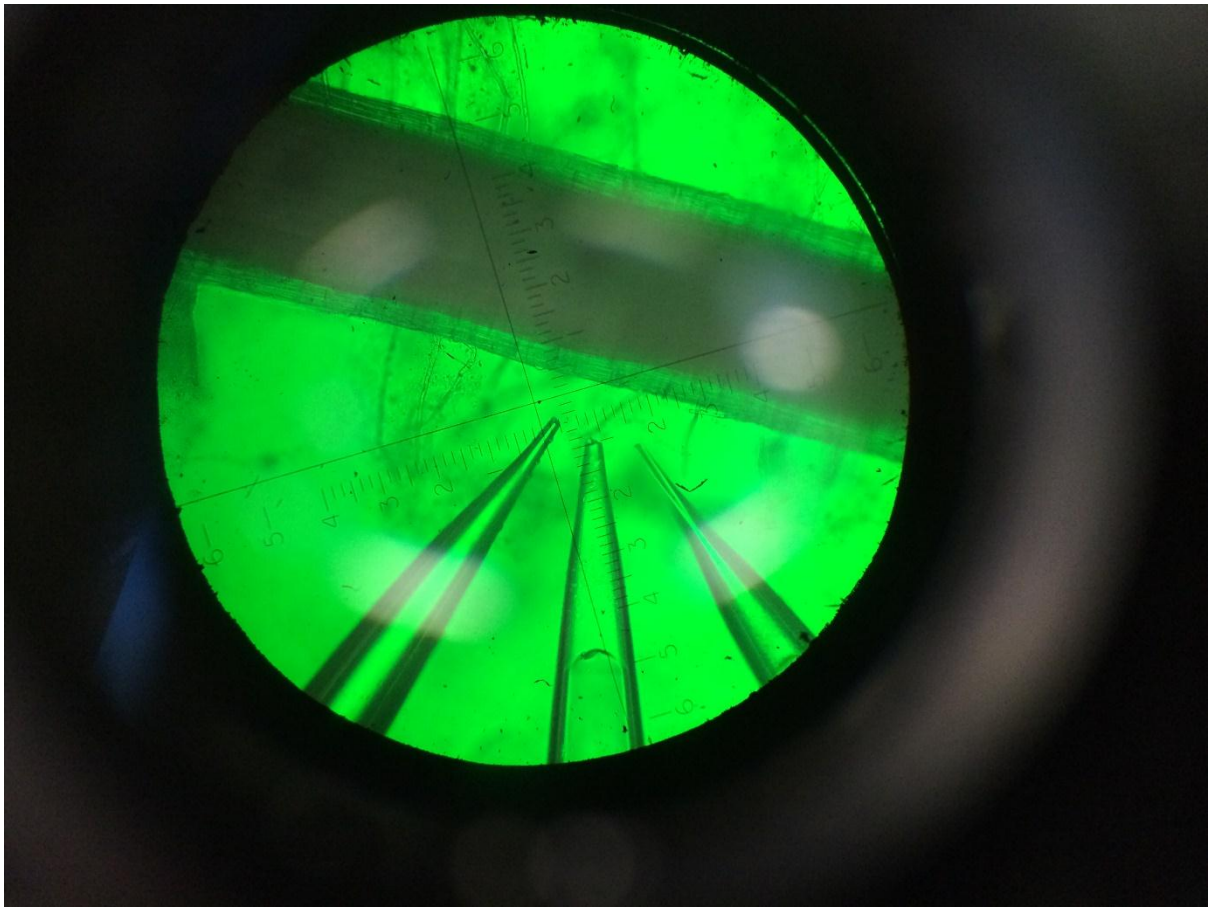

Figure 3.15. Positioning electrodes at the root tissue. The distance was controlled using a graticule inserted in the microscope eye piece.

3.3.7 Data analysis

Flux file was calculated using the MIFE software assuming cylindrical geometry of the root and also using specific parameters of the object in study (root radius, **z**, and initial distance between the root and the electrodes, **u**, M1 position). The calculated flux file was transferred to a personal computer and SPSS and Excel were used for data analysis and graphing.

The average data from 6-10 replicated were analyzed by IBM SPSS statistics 20 (IBM, New York, USA). Univariate General Linear Model with Duncan test was used to confirm the significant difference between treatments and varieties. Bivariate correlation was used to determine the significant correlation between the characteristics that had been measured.

Chapter 4: Effects of waterlogging and salinity and their combination on agronomical and physiological characteristic of pot-grown plants

4.1 Introduction

Plant growth is decreased under combined waterlogging and salinity (WL/NaCl) stress. Combined WL/NaCl stress has significantly decreased the net photosynthesis rate (P_N) of the flag leaf of wheat plants after anthesis that could be related to stomatal closure. Further P_N reduction in the same plants was assumed to be associated with chlorosis, damage to the photosystem II (PSII), enhanced lipid peroxidation, and depressed ATP synthesis in the chloroplasts of the flag leaf (Zheng et al. 2009). It is also known that salinity (NaCl) under waterlogging (WL) conditions caused increased Na^+ and Cl^- concentrations and also decreased K^+ concentrations in the plant shoot compared to salinity under drained conditions (Barrett-Lennard 1986, 2003). The relative tolerance to WL/NaCl stress of some plants such as some varieties of *H. marinum*, was found to be associated with the ability to maintain Na^+ and Cl^- 'exclusion' (Malik et al. 2009). Previously it was assumed that the growth decline of plants under WL/NaCl stress was mostly related to the adverse effects of increased Na^+ and Cl^- concentration but recently decreased K^+ concentration also has been found to be important (Barrett-Lennard and Shabala 2013).

Plant biomass was significantly reduced after 4 weeks exposure to saline drained conditions (Munns et al. 1995) and sensitive plants had died by 4 weeks WL/NaCl stress. Therefore, 15 days' stress was chosen to study and compare the effects of the combined stresses with each stress separately. In this study, the physiological characteristics of 12 barley varieties contrasting in salinity stress tolerance were investigated in response to separate and combined stresses of NaCl and WL. The salinity concentration used was 250mM NaCl because barley plants (glycophytes) are sensitive to salt concentrations above 100-200 mM (Maggio et al. 2001).

This chapter is specifically aimed at addressing three main questions. Firstly, whether the combined WL/NaCl effects are synergistic or additive compared to either separate stress;

secondly, to examine if the effects of WL/NaCl stresses on barley plants are mostly determined by WL or NaCl effects; and thirdly, to study if K^+ or Na^+ ionic relations explain the tolerance of barley varieties to WL/NaCl stresses.

4.2 Results

4.2.1 Damage Index

The damage index scoring system was used to evaluate the overall effects of salinity (NaCl), waterlogging (WL) and their combination (WL/NaCl) on the growth and agronomical characteristics of 12 barley varieties, based on the extent of chlorosis and necrosis in the shoot. The 0 to 10 scaling system was used; with grade 0 given to plants showing no visual symptoms of stress and 10 representing the dead plant. Plants exposed to WL/NaCl stress showed the highest damage index compared to control, NaCl and WL stressed plants (Figures 4.1 and 4.2)

Barley plants under 15 days of NaCl stress expressed much weaker signs of damage compared to WL and WL/NaCl. The most sensitive variety to salinity was Naso Nijo with a damage index of 3, followed by Gairdner and ZUG403 with a damage index of 2, while all other varieties showed minimal visual symptoms of damage (Figure 4.1 A).

Amongst the plants exposed to 15 days of WL stress, the most sensitive variety was Naso Nijo with a damage index of 5, followed by Gebeina, ZUG403, Gairdner and Yerong with a damage index of 4. Franklin and CM72 were found to be the most tolerant varieties to WL with a damage index of 2 (Figure 4.1 B).

The most dramatic changes in damage index happened under WL/NaCl stress. ZUG403 had the highest damage index of 9, with most parts of the plants being dead; followed by Naso Nijo, YSM1 and Gairdner with a damage index of 8. ZUG293 was found to be the most tolerant to WL/NaCl with a damage index of 5, followed by Yerong and YU6472 with a damage index of 6 (Figure 4.1 C). (Figure 4.1 C). Plants changes in damage index under WL/NaCl stress was much more severe than the added damages index of each stress separately (Figure 4.3).

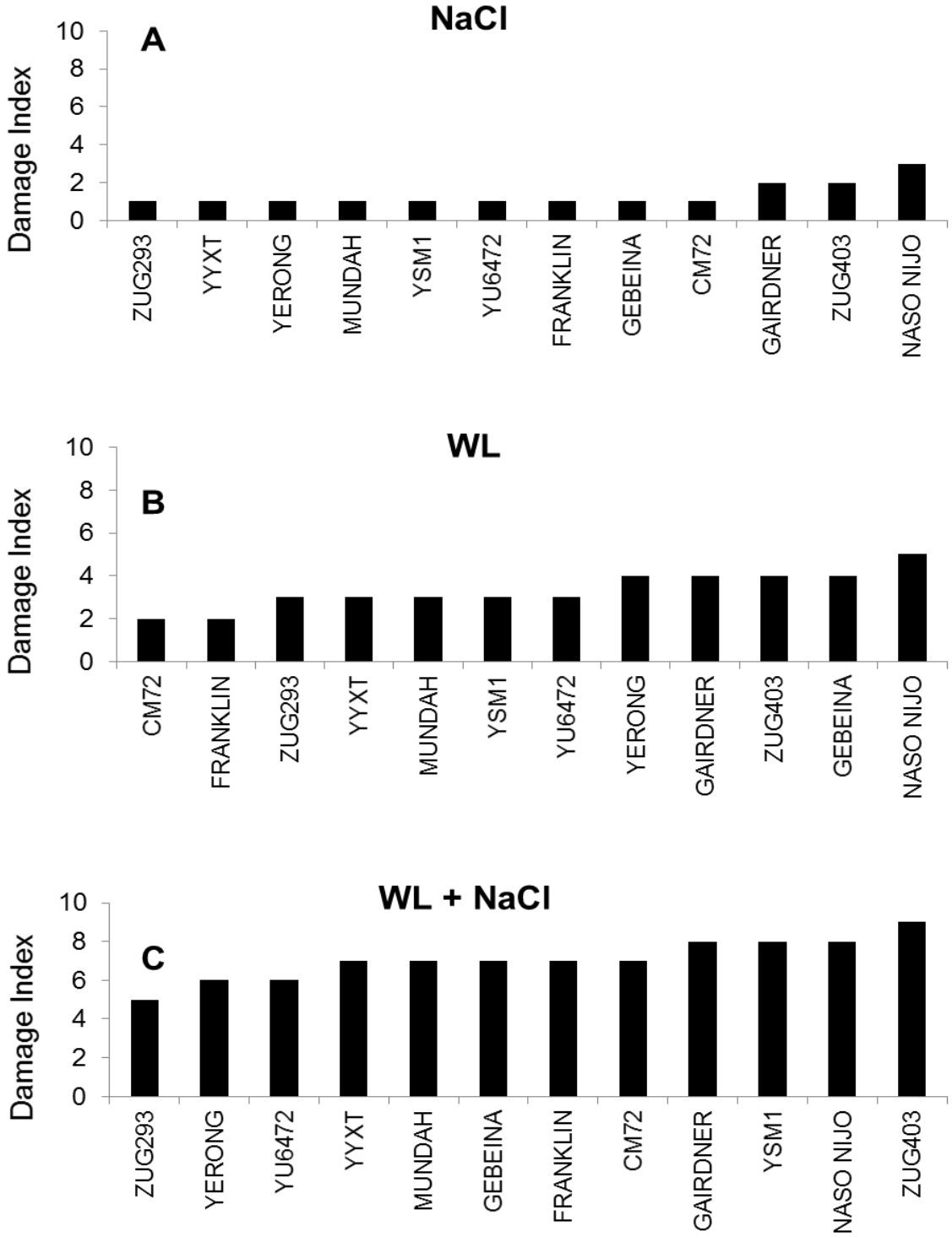

Figure 4.1. Damage Index of 12 barley varieties under separate NaCl and WL stresses and their combination. eight day old seedlings were subjected to one of four treatments; Control (No NaCL, well drained), NaCl (irrigated by 250mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 250mM NaCL solution by 1 cm). Plants damage index was assessed on day 15 of treatment.

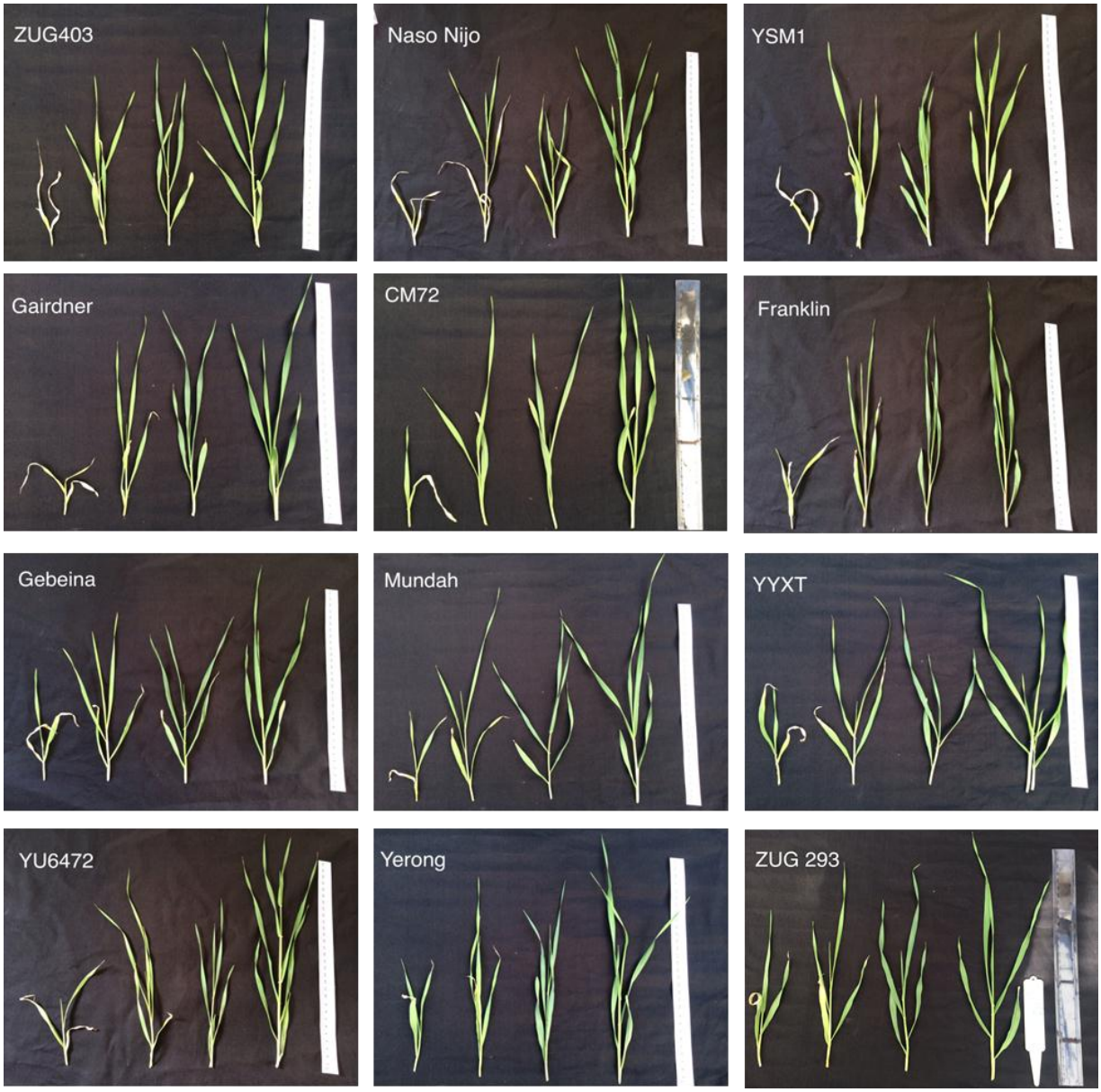

Figure 4.2. Shoot symptoms of the selected 12 barley varieties under non-saline well-drained conditions, 250mM NaCl well-drained, non-saline waterlogged and saline waterlogged conditions after 15 days stress, from right to left respectively.

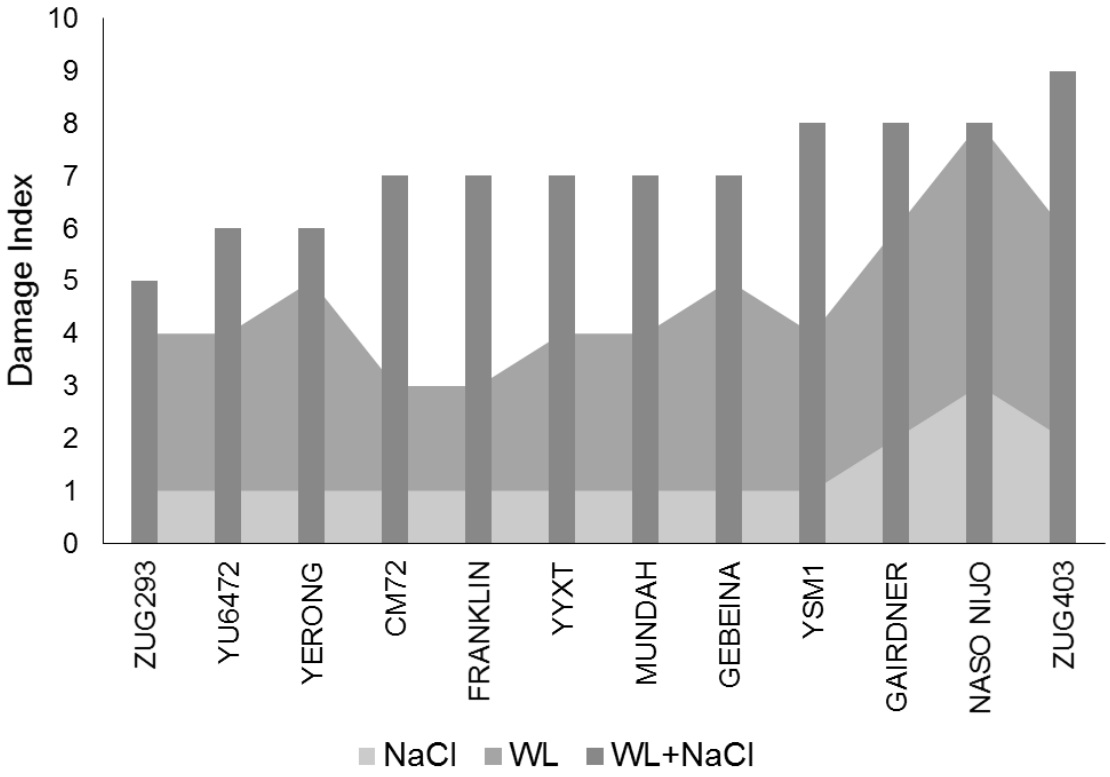

Figure 4.3. Damage index of selected 12 barley varieties under combined WL/NaCl stress compared with the sum of separate NaCl and WL stresses damage index.

The results from correlational analysis between the damage index of barley plants under separate and combined NaCl and WL stresses in the current study and Lit damage index are presented in Figure 4.4. Lit damage index refers to our lab's previous studies of barley varieties damage index to salinity under 300mM NaCl after 40d (Wu et al. 2014).

There is a good correlation between the Lit damage index and the observed damage index in the current experiment under saline conditions (R^2=0.84). The damage index of plants under WL showed lower correlation with the Lit damage index compared to salinity (R^2 =0.45). The damage index of plants under WL/NaCl stress showed the same ratio of WL correlation with the Lit damage index (R^2=0.46) (Figure 4.4).

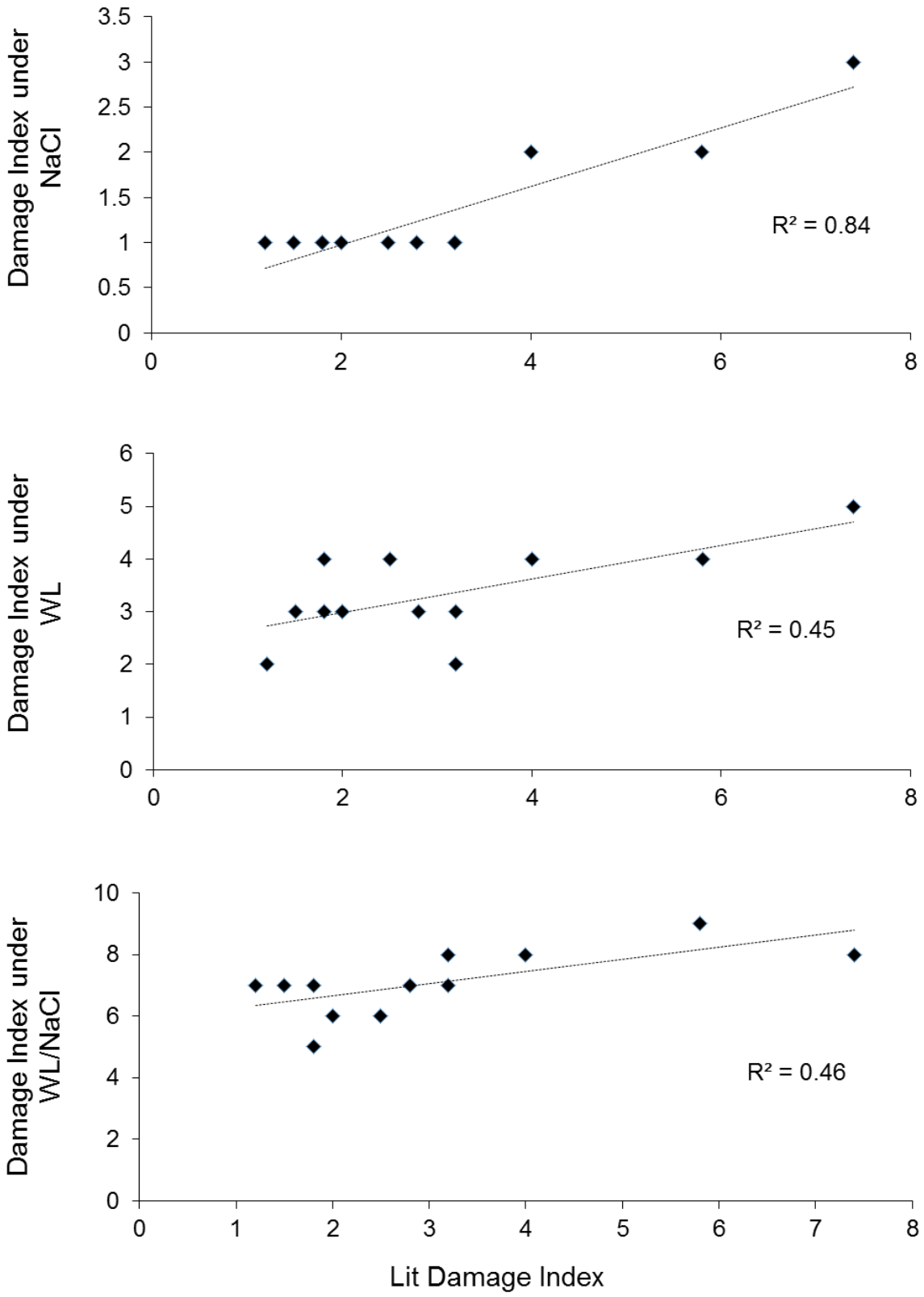

Figure 4.4. Correlation between observed damage index in the current study and Lit damage index (damage index of the barley varieties under saline conditions (Wu. et al 2014)). Each point represents a separate variety of the selected 12 varieties of barley ranging from sensitive to tolerant to salinity under separate and combined stresses of salinity and waterlogging. For growth conditions and details of treatments refer to Material and Methods section.

4.2.2 Plant Growth Performance

Plant growth was significantly ($P<0.001$) reduced by all stress treatments after two weeks of stress. Plants under saline conditions had the least biomass reduction compared to plants under WL and WL/NaCl. All 12 varieties showed the most severe effect on growth under WL/NaCl stress. Shoot biomass under WL/NaCl was reduced by about three to four fold for YYXT, ZUG293 and Yerong, while it reduced the biomass by about ten to twenty fold for ZUG403 and Gairdner (Figure 4.5).

The average shoot fresh weight (FW) of 12 barley varieties were reduced by 87% to 55% relative to control under NaCl treatment, Gebeina and Gairdner had the least and most reduction in FW, respectively. For dry weight (DW) the reduction was between 90% and 56% with YYXT being the least reduced and ZUG403 being the most reduced (Figure 4.5 A-B). The average shoot FW was reduced by 71 to 39% relative to control under WL treatment with Naso Nijo and Mundah having the least and most reduction respectively. The relative DW in the same treatment was 66% to 36% for Franklin and ZUG403, respectively. WL/NaCl stress had the most severe effect on the biomass. The relative FW under WL/NaCl reduced by 42% to 8% respectively for the varieties of YYXT and YSM1. The relative DW of the same treatment reduced by 38% to 9% of the control for the same varieties of YYXT and both ZUG403 and YSM1 (Table 4.1).

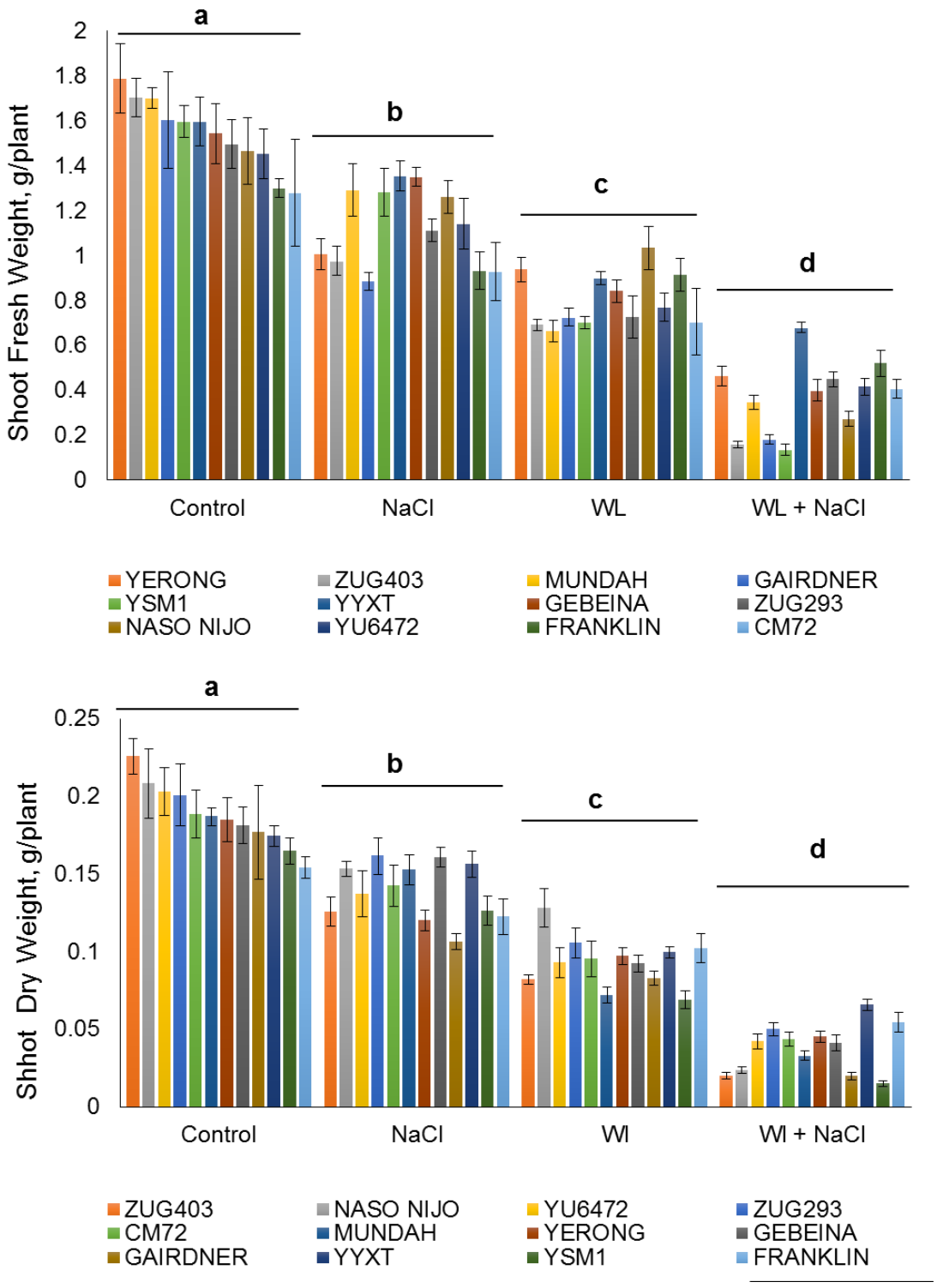

Figure 4.5. Effects of separate and combined stresses of salinity and waterlogging on growth of selected 12 barley varieties in potting mix. 8 day old seedlings were subjected to one of the four treatments; Control (No NaCL, well drained), NaCl (irrigated by 250mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 250mM NaCL solution). Plants were harvested after 15 days stress for biomass measurements. The lower case letters indicate the significant difference between treatments (averaged for all 12 varieties at $P<0.01$, the error bars indicate the standard error of all replicated for each treatment/variety

Table 4.1. Effects of separate and combined stresses of salinity and waterlogging on growth of selected 12 barley varieties in potting mixture relative to their control (%). 8 day old seedlings were subjected to one of the four treatments; Control (No NaCL, well drained), NaCl (irrigated by 250mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 250mM NaCL solution). Plants were harvested after 15 days of treatment for biomass measurements.

Cultivar/ Treatment	Relative Shoot Fresh Weight (% per Control)			Relative Shoot Dry Weight (% per Control)		
	NaCl	WL	WL/NaCl	NaCl	WL	WL/NaCl
ZUG293	74%	49%	30%	80%	53%	25%
CM72	73%	55%	32%	75%	50%	23%
YYXT	85%	56%	42%	90%	57%	38%
YERONG	56%	52%	26%	65%	53%	24%
MUNDAH	76%	39%	20%	82%	38%	18%
GAIRDNER	55%	45%	11%	60%	47%	11%
ZUG403	57%	41%	9%	56%	36%	9%
GEBEINA	87%	55%	26%	89%	51%	23%
YSM1	80%	44%	8%	77%	42%	9%
YU6472	79%	53%	29%	68%	46%	21%
FRANKLIN	72%	70%	40%	79%	66%	35%
NASO NIJO	86%	71%	19%	74%	62%	11%

The Pearson correlation was used to determine relationship between fresh and dry weight of the control and stressed plants. The correlation of fresh and dry weight of the plants under combined stresses with control, NaCl and WL treated is presented in Figure 4.6. Fresh and dry weight of barley varieties under WL/NaCl stress is more correlated with waterlogged plants ($p<0.01$) compared to NaCl treated plants ($p<0.01$ for dry weight and $p<0.05$ for fresh weight) (Table 4.2 and Figure 4.6)

Control plant biomass was more correlated to plants under saline conditions for fresh weight ($p<0.05$) and more correlated to plants under WL conditions for dry weight ($p<0.01$) Plants treated by NaCl and WL showed a good correlation for both fresh and dry weight ($p<0.01$).

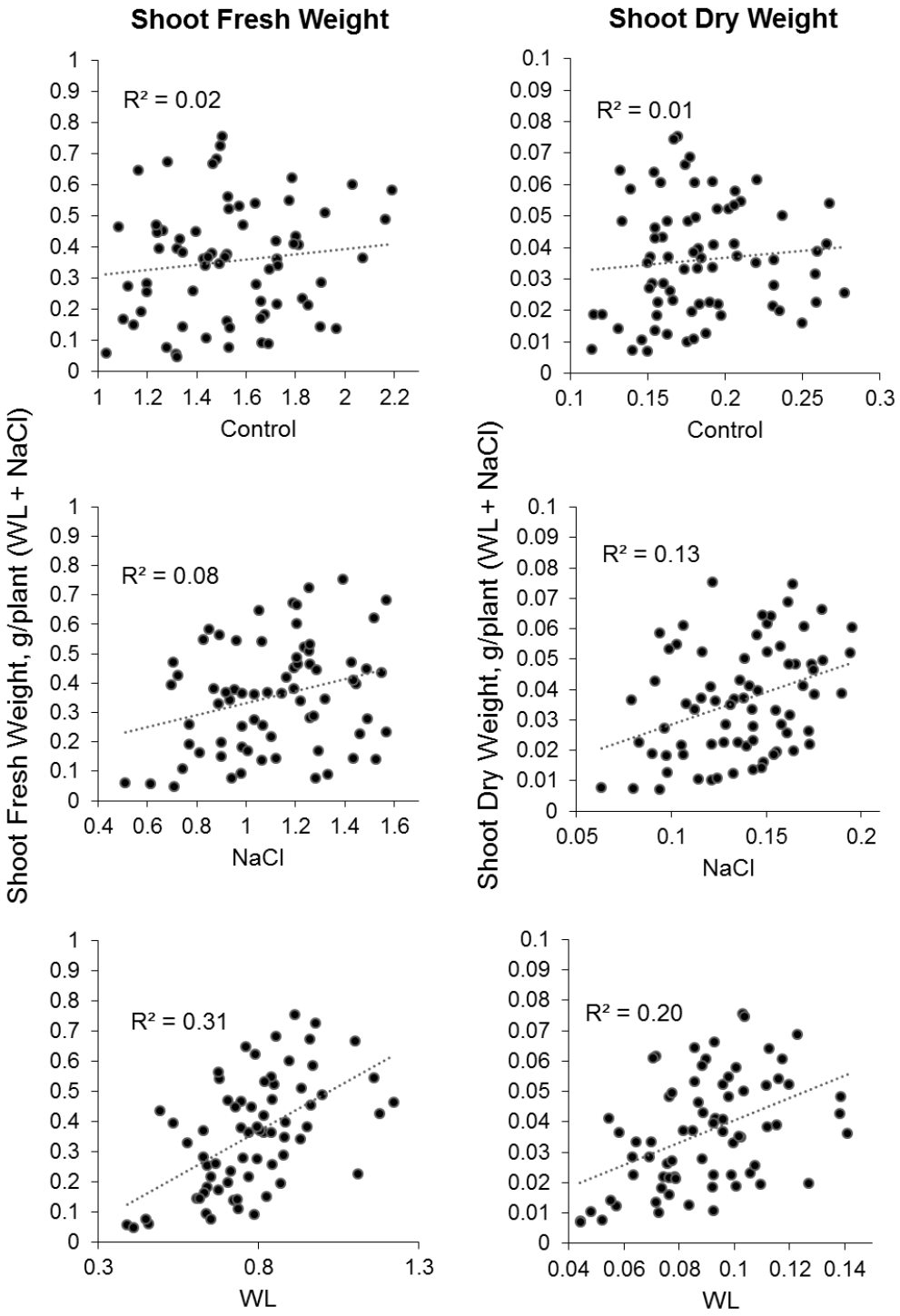

Figure 4.6. Correlation between shoot fresh and dry weight of barley varieties under combined stresses of salinity and waterlogging with control, Nacl and Wl stressed plants

Table 4.2. Correlation between shoot fresh and dry weight of 12 barley varieties under WL/NaCl stress with control, NaCl and WL stressed plants

		Fresh Weight		
		NaCl	WL	WL/NaCl
Fresh Weight	Control	0.247*	0.133	0.126
	NaCl		0.305**	0.283*
	WL			0.554**

		Dry Weight		
		NaCl	WL	WL/NaCl
Dry Weight	Control	0.287*	0.313**	0.093
	NaCl		0.383**	0.361**
	WL			0.451**

4.2.3 Chlorophyll Content

10 days of WL and WL/NaCl treatment lead to a significant reduction in chlorophyll content ($P < 0.01$, averaged for all 12 varieties) while there was no significant effect on the plants under salinity only conditions compared to the control (averaged for all 12 varieties, see Figure 4.7). The average chlorophyll content SPAD value of the plants under NaCl treatment after 10 days of treatment was 98%-114% relative to control, Gebeina and YYXT respectively had the most and least reduction. All the varieties except Gebeina showed higher chlorophyll content SPAD value under saline conditions compared to control plants. The average chlorophyll content under WL stress relative to control after 10 days ranged from 74%-94%, Naso Nijo and Yerong had the most and least reduction, respectively. Plants under WL/NaCl had 2%-78% of chlorophyll content SPAD value relative to control. Gebeina and Yerong had the most and least reduction, respectively (Table 4.3).

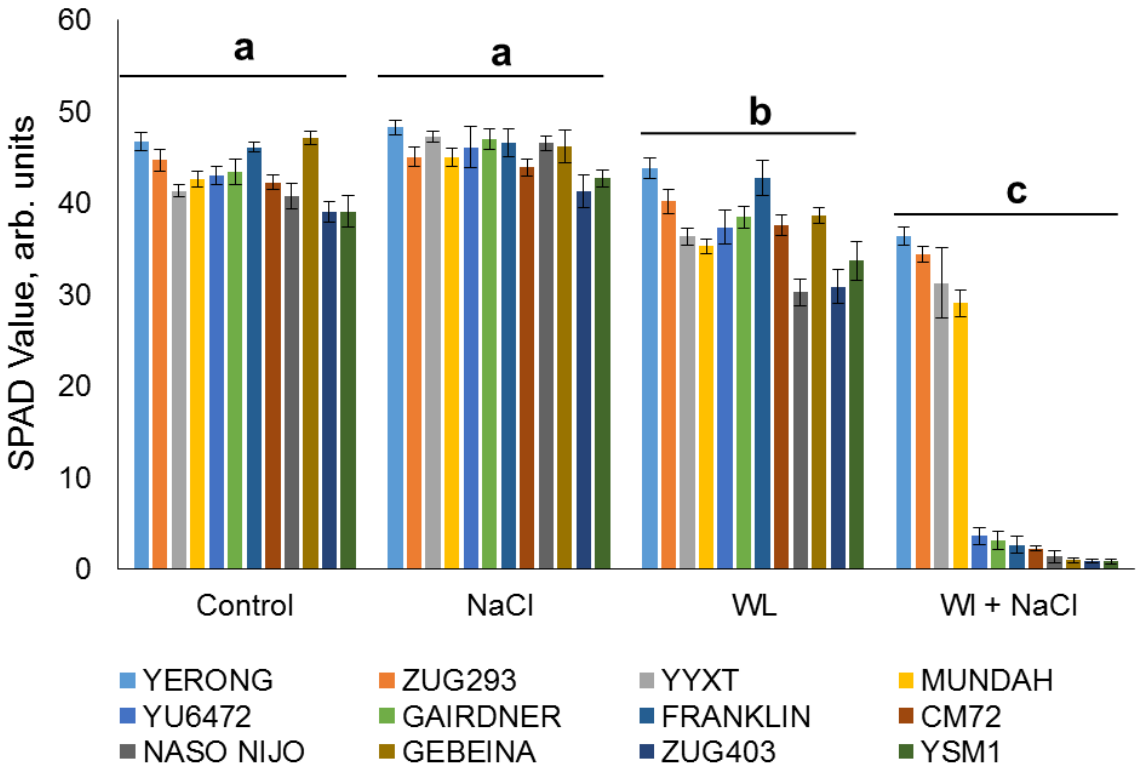

Figure 4.7. Effects of separate and combined salinity and waterlogging stresses on chlorophyll content (SPAD values) of selected 12 varieties of barley from a range of sensitive to tolerant to salinity. Measurements were taken 10 days after the treatment onset. For growth conditions and details of treatments refer to Material and Methods section. Different lower case letters indicate the significance of differences between treatments (averaged for all 12 varieties) at $P < 0.01$, the error bars indicate the standard error of all replicated for each treatment/variety

Table 4.3. The minimum and maximum chlorophyll content SPAD value of 12 varieties of barley under 10 days separate and combined stresses of NaCl and WL relative to the control (%) and minimum and maximum chlorophyll content SPAD value of selected 4 varieties of barley under 14 days separate and combined stresses of NaCl and WL relative to the control (%)

	SPAD (10 Days Stress)	**SPAD (14 Days Stress)**
NaCl relative to control	98% - 114%	112% - 125%
WL relative to control	74% - 94%	63% - 92%
WL/NaCl Relative to control	2% - 78%	5% - 83%

The results obtained from correlational analysis of SPAD value showed that there was not a good correlation between SPAD value of WL/NaCl treated plants with control, NaCl and WL treated plants. While there is a good correlation between control, NaCl and WL stressed plants SPAD value (Figure 4.8).

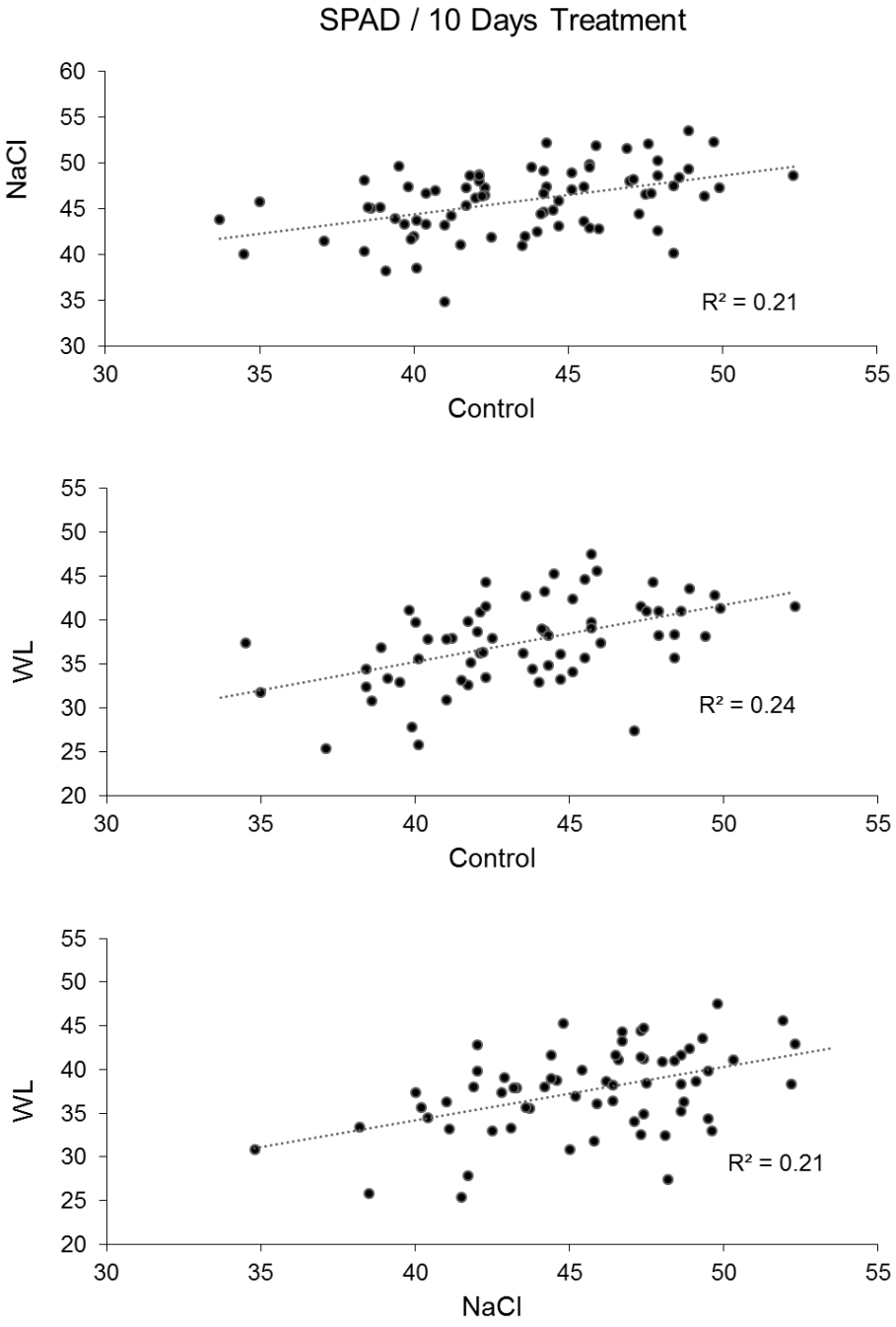

Figure 4.8. Correlation between chlorophyll content (SPAD value) of 12 barley varieties under control conditions and separate stresses of salinity and waterlogging. 8 day old seedlings were subjected to one of the four treatments. Plant chlorophyll content was measured on-site on day 10 of treatment. For growth conditions and details of treatments refer to Material and Methods section.

A closer look at the SPAD value of WL/NaCl stressed plants shows a big difference between two groups of varieties; first, varieties with less than 10% of SPAD value relative to control and second, varieties with more than 60% of SPAD value relative to control. Yerong, ZUG293, YYXT and Mundah were the varieties that showed the highest SPAD value amongst the 12 varieties (more than 60%). These four varieties were monitored for their SPAD value in day 14 of treatment. The average chlorophyll content SPAD value of the selected varieties under saline conditions after 14 days ranged between 112%-125% relative to the control, Mundah and YYXT had the most and least reduction respectively. Average SPAD value of the plants under WL conditions ranged between 63%-92% relative to the control, YYXT and Yerong had the most and least reduction respectively. 14 days WL/NaCl stress had the most effect on chlorophyll content of the selected varieties ranging between 5%-83% average SPAD value relative to the control Mundah and Yerong had the most and least reduction respectively (Figure 4.9).

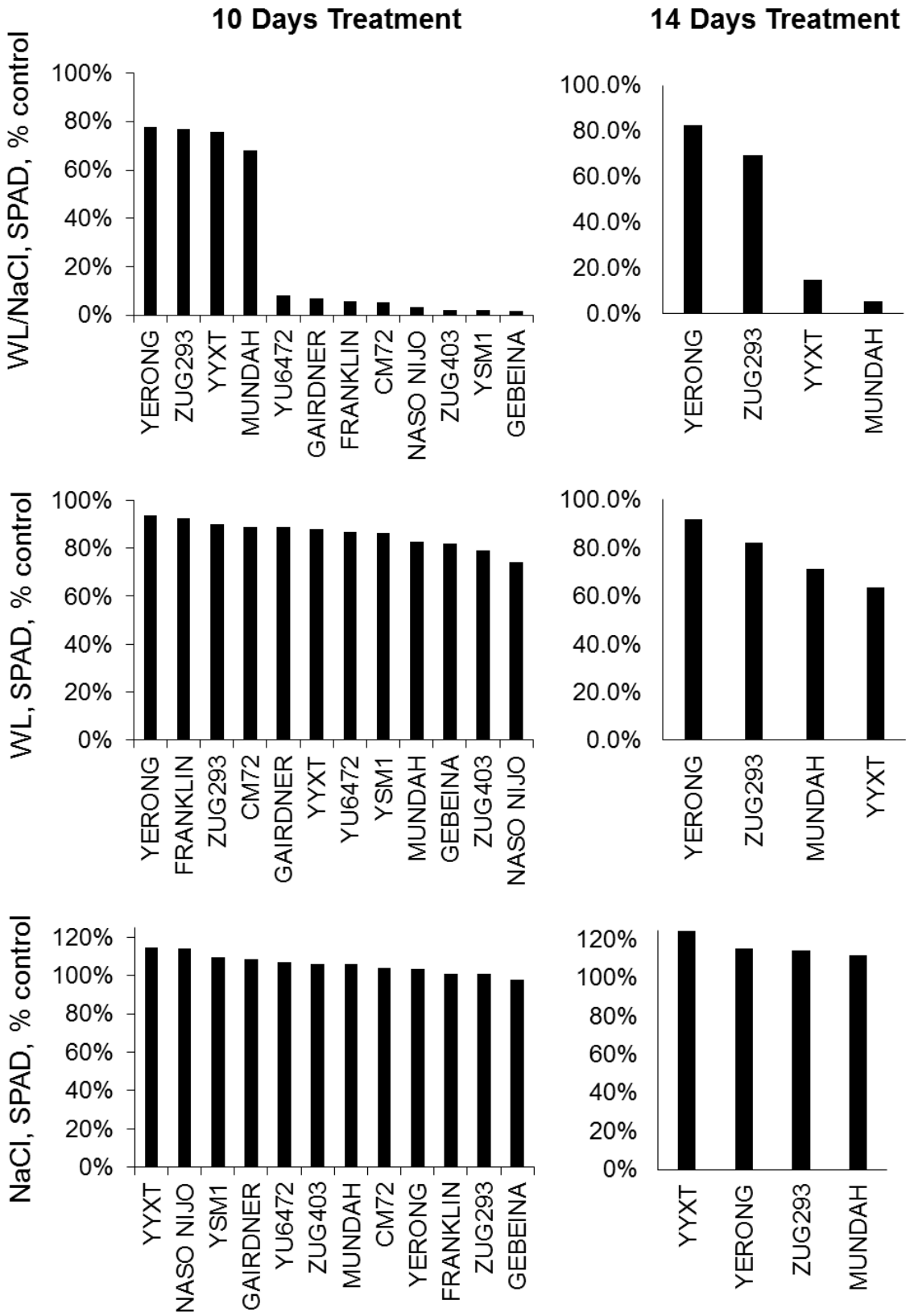

Figure 4.9. Effects of separate and combined salinity and waterlogging stresses on chlorophyll content (SPAD values) of selected 12 varieties of barley relative to their control (%). SPAD value measurements were taken 10 and 15 days after the treatment onset. For growth conditions and details of treatments refer to Material and Methods section.

The twelve varieties were divided to two groups for further correlational purposes. First, the varieties which had less than 10% SPAD value under WL/NaCl relative to control; YU6472, Gairdner, Franklin, CM72, Naso Nijo, ZUG403, YSM1 and Gebeina (sensitive to WL/NaCl) and second, the varieties which had more than 60% SPAD value under WL/NaCl relative to control; Yerong, ZUG293, YYXT and Mundah (tolerant to WL/NaCl). A new correlational analysis was applied to observe the difference in the trends of both groups (Figure 4.10 and Tables 4.4-6). As it is shown below, the tolerant group of varieties showed strong correlation between SPAD values of WL/NaCl stressed plants and WL plants after both 10 and 14 days (Figure 4.10). They also showed strong correlation between WL/NaCl and NaCl stressed plants after 14 days. While the sensitive group of varieties did not show a good correlation between WL/NaCl and WL, NaCl or control plants. The sensitive group of varieties showed a strong correlation between control, WL and NaCl. While the tolerant group of varieties showed no correlation between WL, NaCl and also WL/NaCl after 10 days but they showed good correlation between NaCl stresses plants and control (Table 4.6 and Figure 4.10).

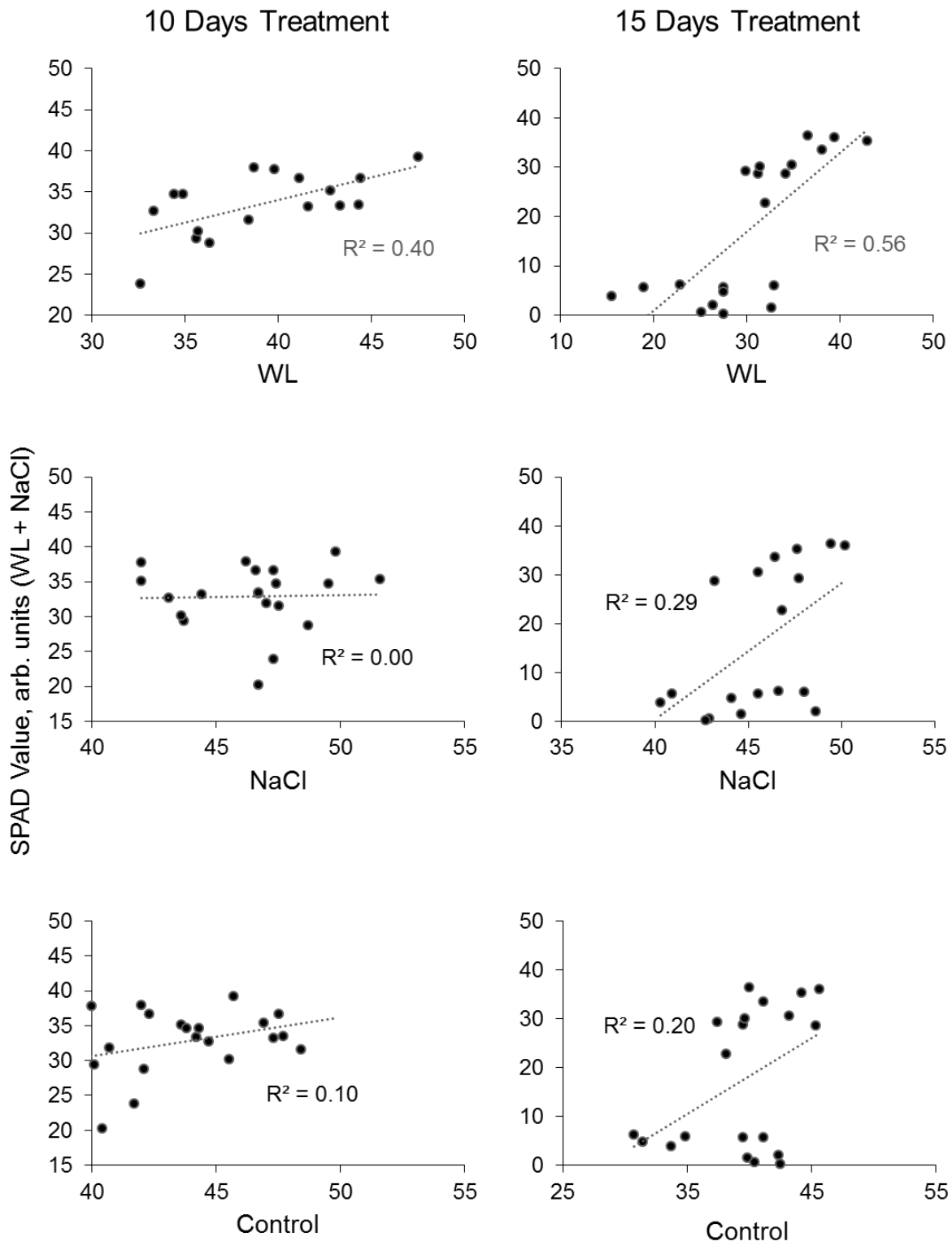

Figure 4.10. Comparing the correlation between chlorophyll content (SPAD value) of different barley varieties under separate and combined stresses of salinity and waterlogging after 10 and 15 days of treatment for selected barley varieties when their oldest leaf survived for 15 days (tolerant varieties to WL/NaCl).

Table 4.4. The correlation analysis of all 12 varieties of barley for SPAD value

		SPAD/10 Days Treatment/all varieties		
		Control	NaCl	WL
SPAD	NaCl	0.455**		
	WL	0.495**	0.458**	
	WL/NaCl	0.057	0.099	0.234

Table 4.5. The correlational analysis of sensitive varieties to WL/NaCl stress after 10 days of separate and combined stresses of NaCl and WL

		SPAD/10 Days Treatment/ Sensitive varieties		
		Control	NaCl	WL
SPAD	NaCl	0.487**		
	WL	0.523**	0.537**	
	WL/NaCl	-0.018	-0.020	0.005

Table 4.6. The correlational analysis of tolerant varieties to WL/NaCl stress after 10 and 14 days of separate and combined stresses of NaCl and WL

	SPAD/10 Days Treatment			**SPAD/14 Days Treatment**		
	Control	NaCl	WL	Control	NaCl	WL
NaCl	0.280			0.086		
WL	0.380	0.085		0.489*	0.559**	
WL/NaCl	0.324	0.033	0.629**	0.447*	0.541*	0.746**

4.2.4 Chlorophyll Fluorescence

The maximum photochemical efficiency of PSII (Chlorophyll Fluorescence Fv/Fm Value) of the plants was highly reduced by 15 days WL/NaCl and WL stresses but it was not reduced significantly by NaCl stress. The average chlorophyll fluorescence under NaCl stress after 15 days was 101-103% relative to control, Franklin and Mundah respectively had the most and least reduction. The selected barley varieties under NaCl treatment showed higher Fv/Fm value compared to control plants. The average Fv/Fm value under WL treatment was 70-100% for Naso Nijo and Yerong respectively. The average Fv/Fm relative to control under WL/NaCl was calculated from 9% to 100% for Gairdner and Yerong (Figure 4.12).

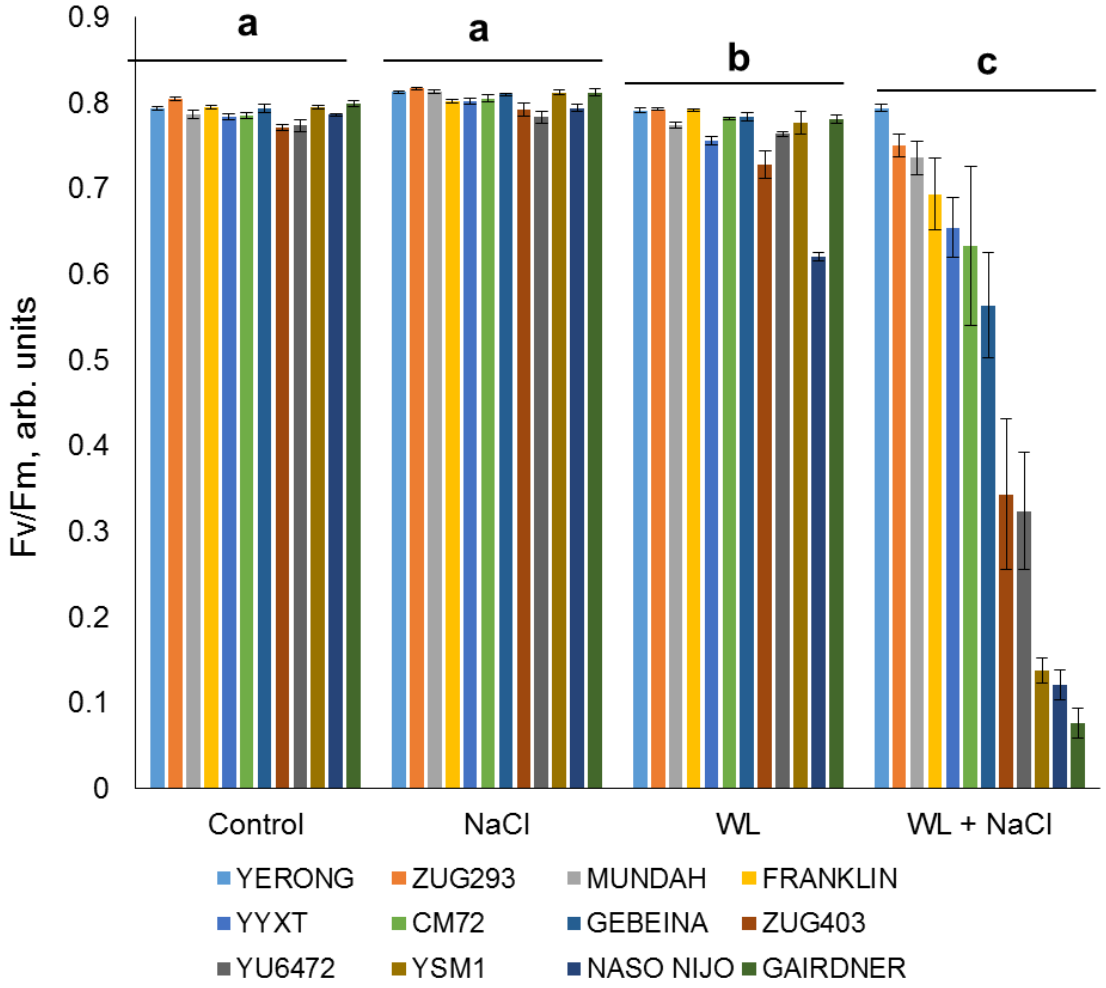

Figure 4.11. Effects of separate and combined salinity and waterlogging stresses on maximum photochemical efficiency of PSII (Fv/Fm chlorophyll fluorescence values) of selected 12 barley varieties. Measurements were taken 15 days after the treatment onset. For growth conditions and details of treatments refer to Material and Methods section. Different lower case letters indicate the significant difference between treatments (averaged for all 12 varieties) at P< 0.01, the error bars indicate the standard error of all replicated for each treatment/variety

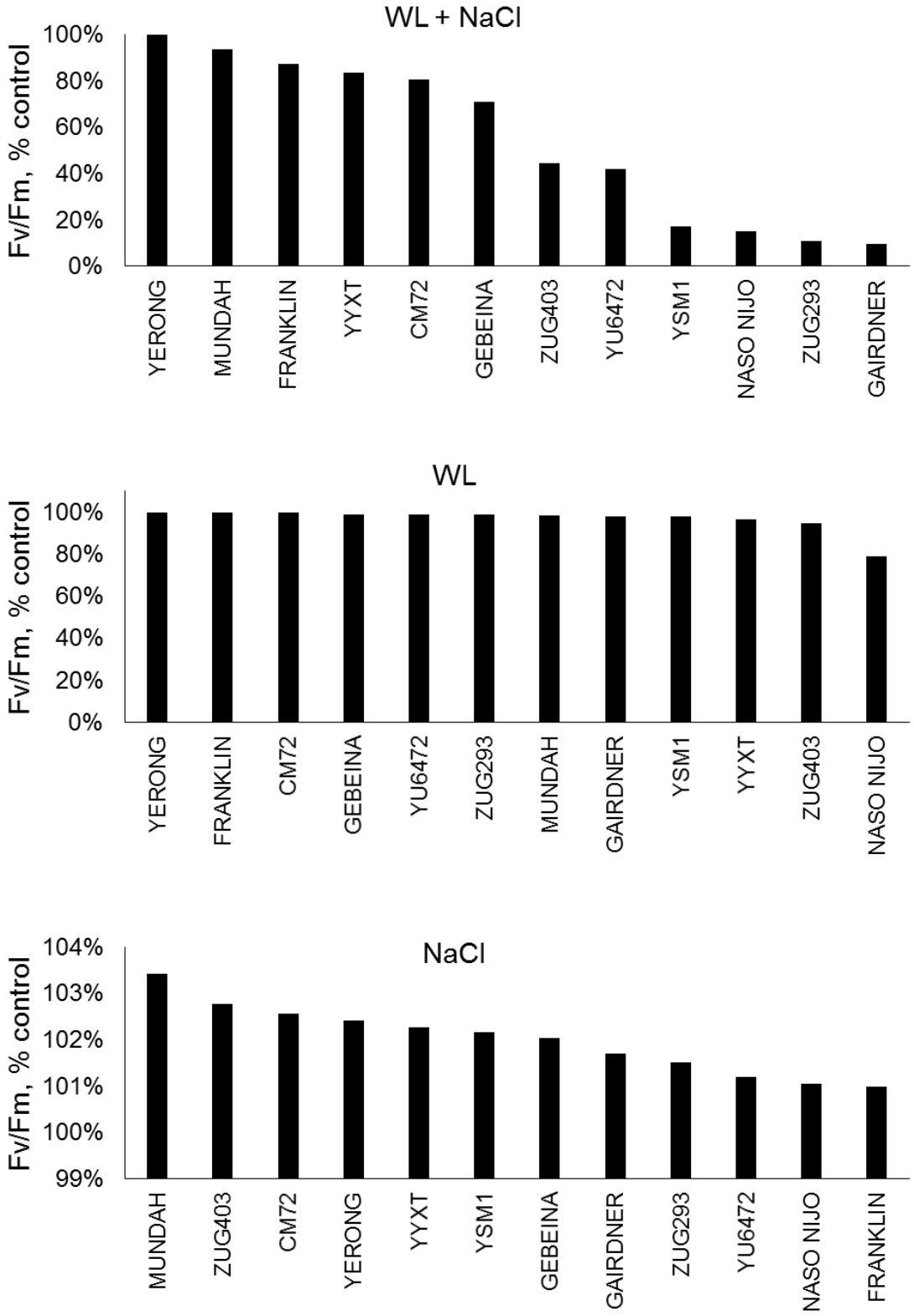

Figure 4.12. Effects of separate and combined salinity and waterlogging stresses on maximum photochemical efficiency of PSII (Fv/Fm chlorophyll fluorescence values) of selected 12 barley varieties relative to control. Measurements were taken 15 days after the treatment onset. For growth conditions and details of treatments refer to Material and Methods section.

The Pearson correlational analysis showed that the Fv/Fm value of WL/NaCl and WL stressed plants were significantly correlated ($p<0.05$). The Fv/Fm value of NaCl and WL stress was significantly correlated with control ($p<0.01$). The highest correlation between Fv/Fm value of the plants under control conditions and stressed plants was for NaCl stressed plants (Figure 4.13).

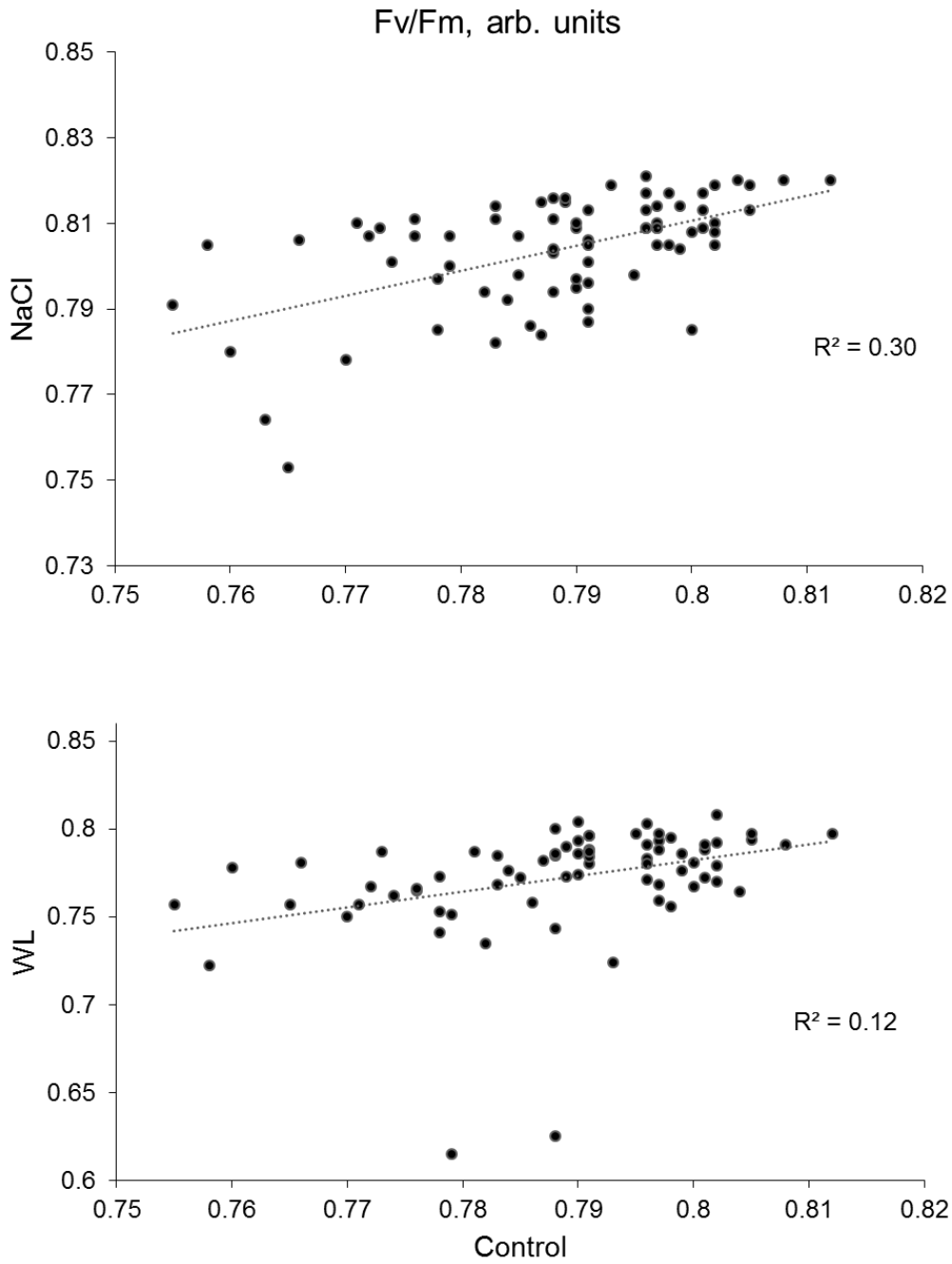

Figure 4.13. Correlation between maximum photochemical efficiency of PSII (Fv/Fm chlorophyll fluorescence values) of barley varieties under salinity and waterlogging stress with plants under drained non-saline conditions

Table 4.7. Correlation between maximum photochemical efficiency of PSII (Fv/Fm chlorophyll fluorescence values) of barley varieties under NaCl, WL, WL/NaCl and control conditions

		Fv/Fm		
		Control	NaCl	WL
Fv/Fm	NaCl	0.551**		
	WL	0.346**	0.180	
	WL/NaCl	0.152	0.235	0.293*

Comparing the correlation of the observed damage index under separate and combined stresses of NaCl and WL with each of the measured characteristics such as shoot FW and DW, chlorophyll content SPAD value and chlorophyll florescence Fv/Fm value shows that Fv/Fm value has the greatest correlation for all three treatments and the highest for WL/NaCl treatment (Table 4.8). It is shown in Figure 4.14 how different varieties based on their tolerance to each of the separate and combined NaCl and WL stresses are ranked based on the measured parameters and it is apparent from the figures that Fv/Fm separated the varieties much more obviously compared to biomass and chlorophyll contents.

Table 4.8. Correlation between damage index of the plants under NaCl, WL and WL/NaCl stress with their shoot fresh and dry weight, chlorophyll content SPAD value and chlorophyll florescence Fv/Fm value

		Damage Index		
		NaCl	WL	WL/NaCl
Shoot Fresh Weight	NaCl	-0.031		
	WL		0.134	
	WL/NaCl			-0.598**

		Damage Index		
		NaCl	WL	WL/NaCl
Shoot Dry Weight	NaCl	-0.006		
	WL		0.123	
	WL/NaCl			-0.635**

		Damage Index		
		NaCl	WL	WL/NaCl
SPAD Value	NaCl	-0.077		
	WL		-0.171	
	Wl/NaCl			-0.650**

		Damage Index		
		NaCl	WL	WL/NaCl
Fv/Fm	NaCl	-0. 254*		
	WL		-0.357**	
	WL/NaCl			-0.751**

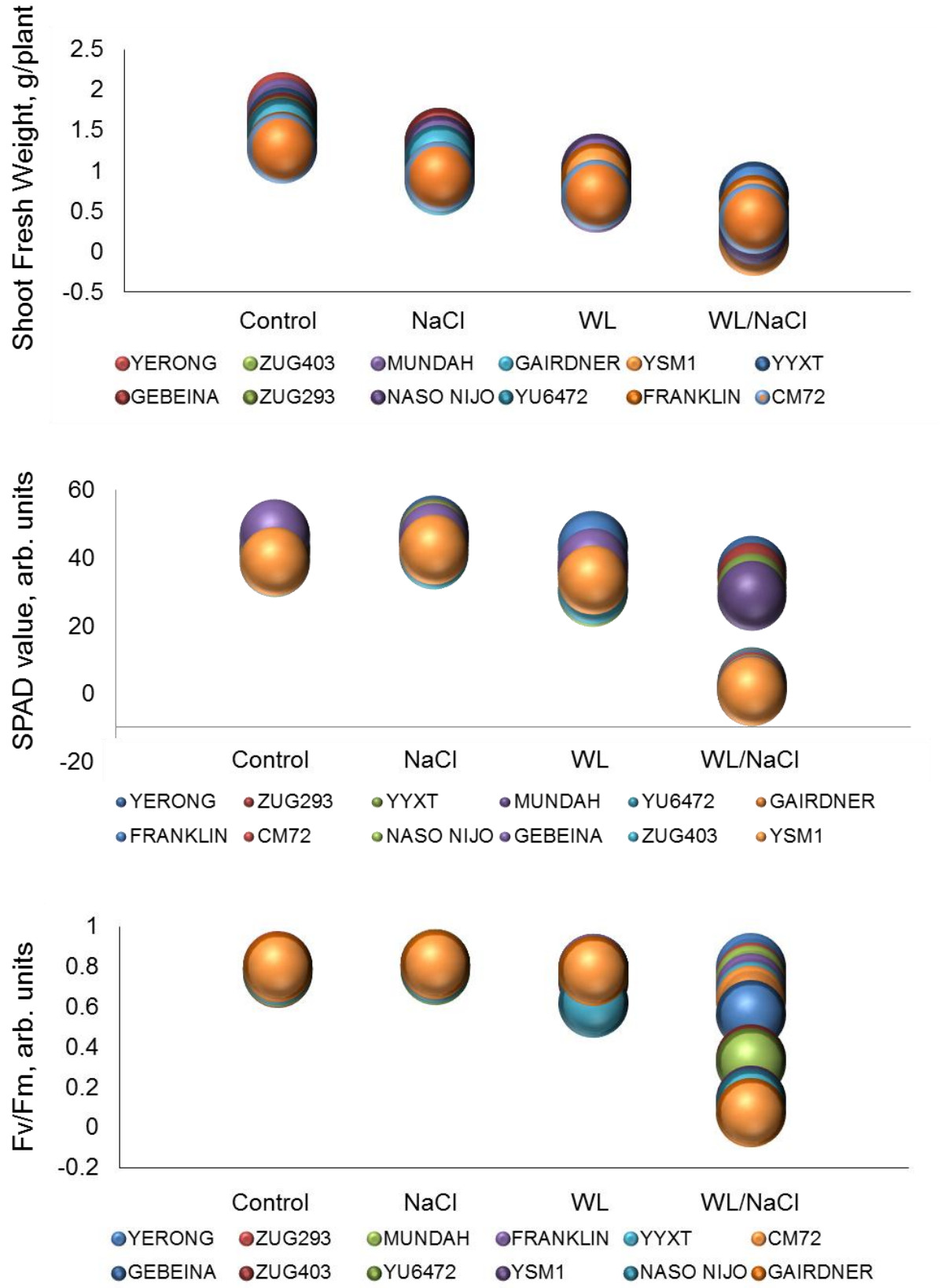

Figure 4.14. Effects of separate salinity and waterlogging stresses and their combination on growth, chlorophyll content (SPAD values) and maximum photochemical efficiency of PSII (Fv/Fm chlorophyll fluorescence values)

4.2.5 Na^+ and K^+ Content

4.2.5.1 Na^+ content

NaCl treatment increased Na^+ content of all barley varieties regardless to their tolerance to salinity. As shown in Figure 4.15, even though plants under WL stress showed slightly more Na^+ content compared to the control, the average Na^+ content for the 6 varieties under control and WL conditions is statistically the same ($P<0.0001$). Plants under WL/NaCl conditions showed higher Na^+ content compared to plants under NaCl conditions. The average shoot Na^+ content for 6 barley varieties after 15 days of NaCl treatment was 11 to 24 fold higher than compared to control conditions, Gairdner and YYXT had the least and most increase respectively. WL/NaCl stresses had the most effect on the all six selected varieties. The average shoot Na^+ content of barley varieties under WL/NaCl conditions was 21 to 66 fold higher than control conditions. Gairdner and ZUG403 had the least and YYXT had the most increase. Na^+ content in WL/NaCl treated plants was 6 to 12 fold higher comparted to plants under NaCl conditions, ZUG403 and ZUG293 had the least and most increase respectively. Plants under WL/NaCl conditions showed 10 to 22-fold higher Na^+ content compared to plants under WL conditions, ZUG403 and YYXT had the least and most increase respectively.

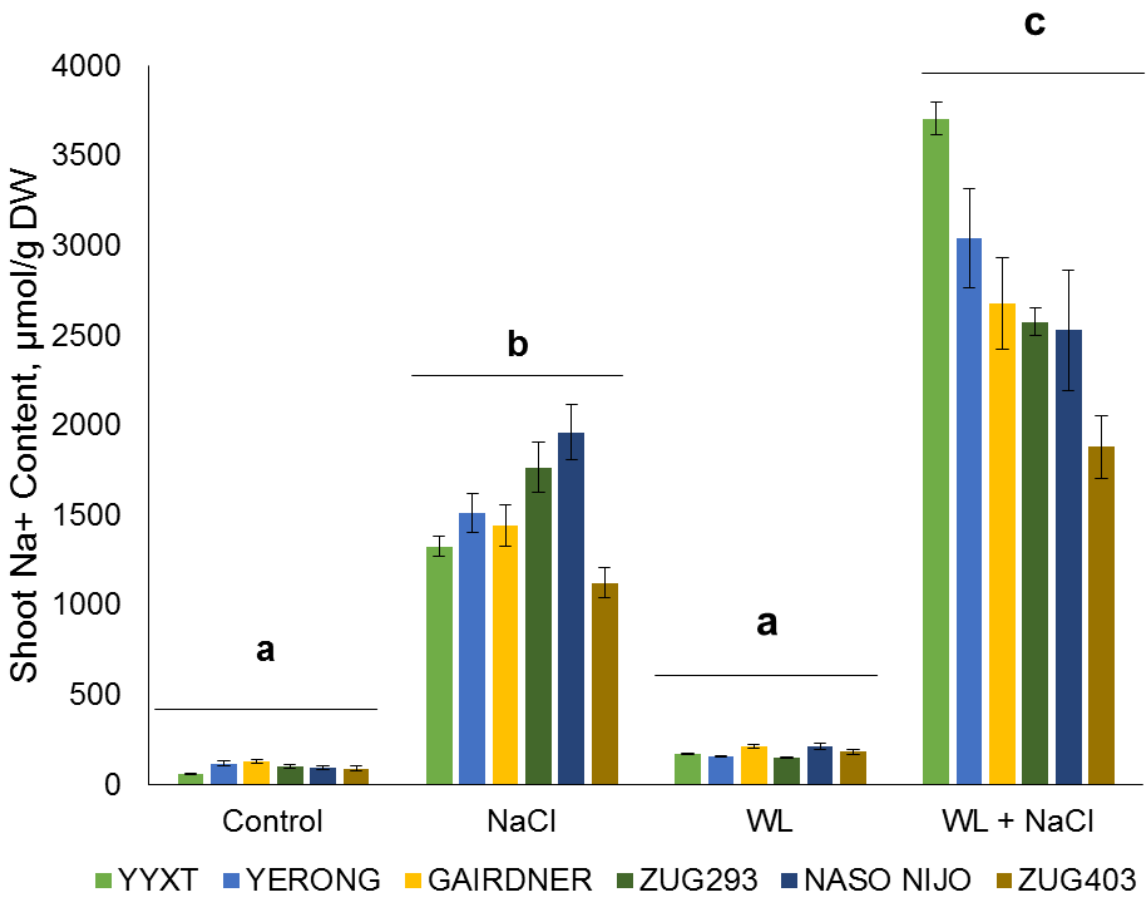

Figure 4.15. Effects of separate and combined salinity and waterlogging stresses on tissue Na+ content of selected 6 barley varieties. Measurements were taken 15 days after the treatment onset. For growth conditions and details of treatments refer to Material and Methods section. Different lower case letters indicate the significant difference between treatments (averaged for all 12 varieties) at P< 0.01, the error bars indicate the standard error of all replicated for each treatment/variety

It was demonstrated that salinity treatment results in high Na^+ intake of the shoot in barley varieties. Varieties sensitive to salinity acquired more Na^+ in the shoot compared to tolerant varieties and as Na^+ replaces the functioning role of K^+ the adverse effects on growth and finally death of the plant is anticipated. WL/NaCl stress was expected to raise Na^+ content and reduce K^+ content in the sensitive varieties and reduce Na^+ content and raise K^+ content for tolerant varieties to salinity, but the results were reversed. Sensitive varieties to salinity such as Gairdner, Naso Nijo, ZUG403 showed lower Na^+ content compared to tolerant varieties to salinity such as ZUG293, Yerong and YYXT. A closer look at the divided groups to tolerant and sensitive varieties to salinity will explain the reason.

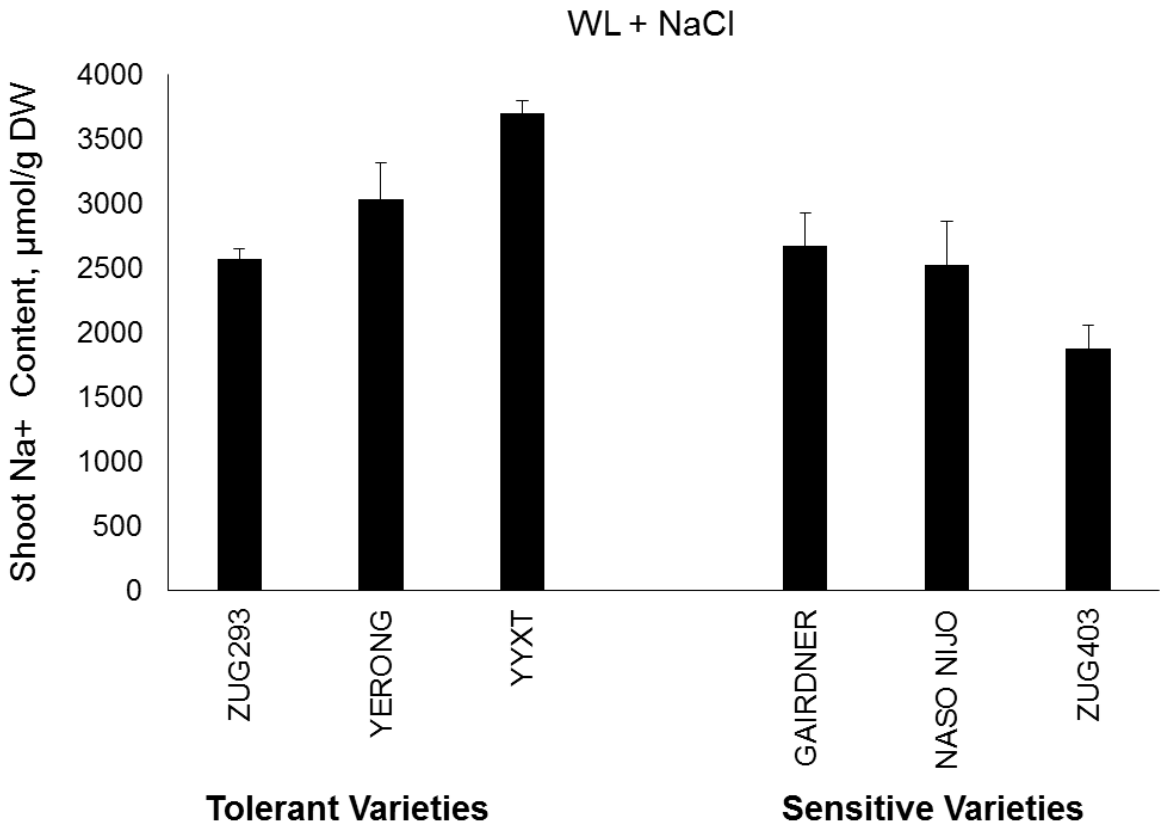

Figure 4.16. Effects of separate and combined salinity and waterlogging stresses on tissue Na^+ content of selected 6 barley varieties divided in two groups of sensitive and tolerant to combined stresses of salinity and waterlogging. Measurements were taken 15 days after the treatment onset. For growth conditions and details of treatments refer to Material and Methods section. the error bars indicate the standard error of all replicated for each treatment/variety

The six selected varieties were divided into two groups; first, tolerant to salinity comprising ZUG293, Yerong and YYXT; second, sensitive to salinity comprising Gairdner, Naso Nijo and ZUG403. Amongst the tolerant varieties to salinity ZUG293 with 2573 mM and YYXT with 3704 mM had the least and most Na^+ content, respectively. Amongst the sensitive varieties to salinity ZUG403 with 210 mM and Gairdner with 2674 mM had the least and most Na^+ content (Figure 4.16).

The Pearson correlation analysis between Lit damage index and shoot Na+ content of plants under control, NaCl, WL and WL/NaCl conditions shows that while WL plants showed a good strong correlation, WL/NaCl plants showed a good negative correlation (Figure 4.17).

To clarify the results, the tolerant group of varieties to NaCl were analysed separately. The tolerant group showed a good positive correlation between damage index and Na^+ content (Pearson correlation P<0.01, R=0.812, (Figure 4.18)) while sensitive varieties showed a negative correlation (Pearson Correlation P<0.01, R= -0.690**) (the result is not shown).

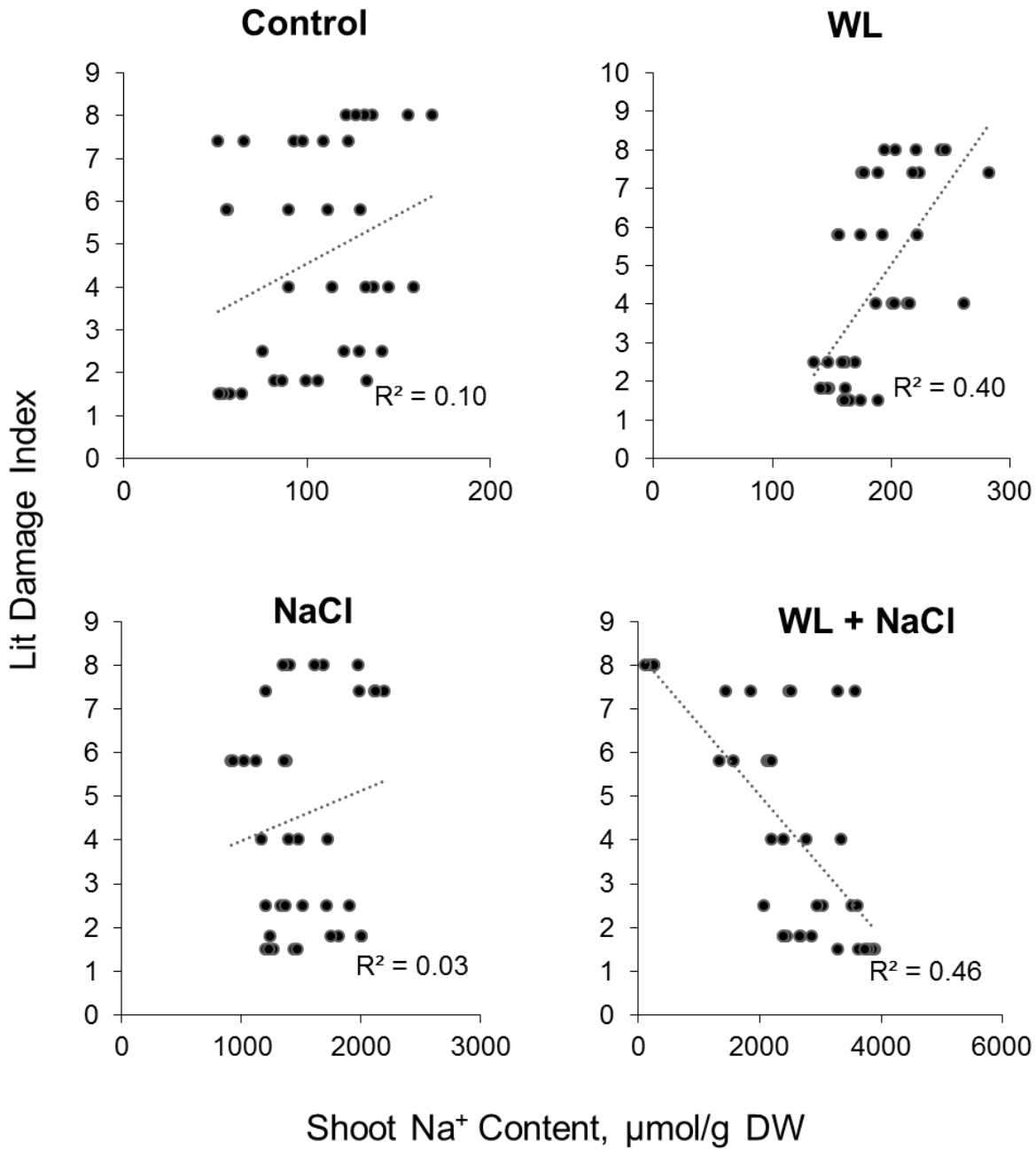

Figure 4.17. Correlation between shoot Na^+ content and damage index of barley varieties grown under separate and combined stresses of salinity and waterlogging and control conditions

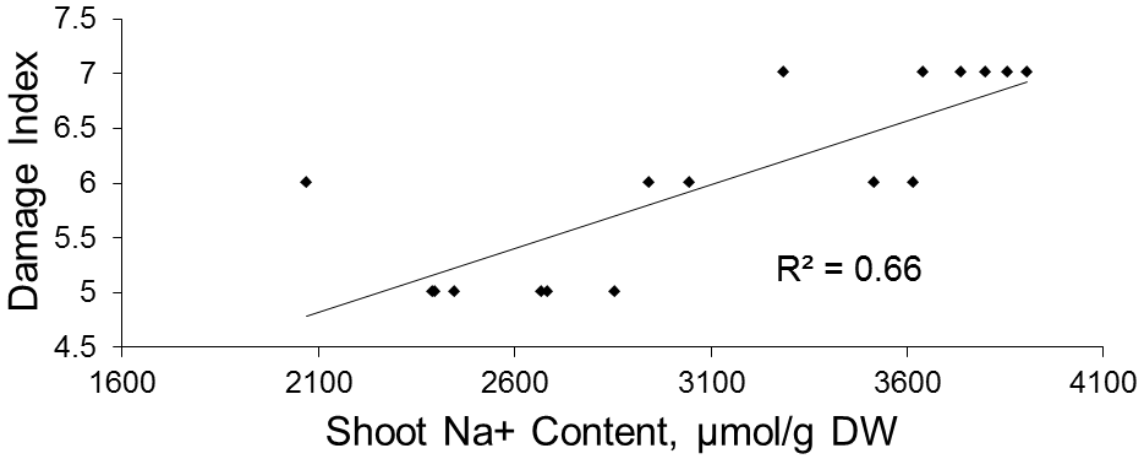

Figure 4.18. Correlation between shoot Na^+ content and damage index of tolerant varieties to salinity ZUG293, Yerong, and YYXT under WL/NaCl stress

4.2.5.2 K^+ content

In a general look at all six varieties, NaCl and WL showed the same small decrease in K^+ content in the shoot, while WL/NaCl stress showed a large decrease in K^+ content (Figure 4.19). The average shoot K^+ content under NaCl treatment after 15 days was 80–108% relative to control, Gairdner and YYXT respectively had the most and least reduction. The same group of varieties under WL stress after 15 days treatment had an average of 82–97% K^+ content reduction relative to control, ZUG403 and Yerong respectively had the most and least reduction. Plants under WL/NaCl showed the widest range of reduction from 9% to 50% relative to control, Gairdner and Yerong had the most and least reduction respectively (Table 4.9).

The six barley varieties were divided in sensitive and tolerant varieties to combined WL/NaCl stress for K^+ content due to the same reason as mentioned for Na^+ content earlier (Tolerant K^+>400mmol). Yerong, ZUG293 and YYXT as tolerant varieties to combined WL/NaCl showed higher K^+ content compared to Gairdner, ZUG403 and Naso Nijo as sensitive varieties to combined WL/NaCl stress (Figure 4.20).

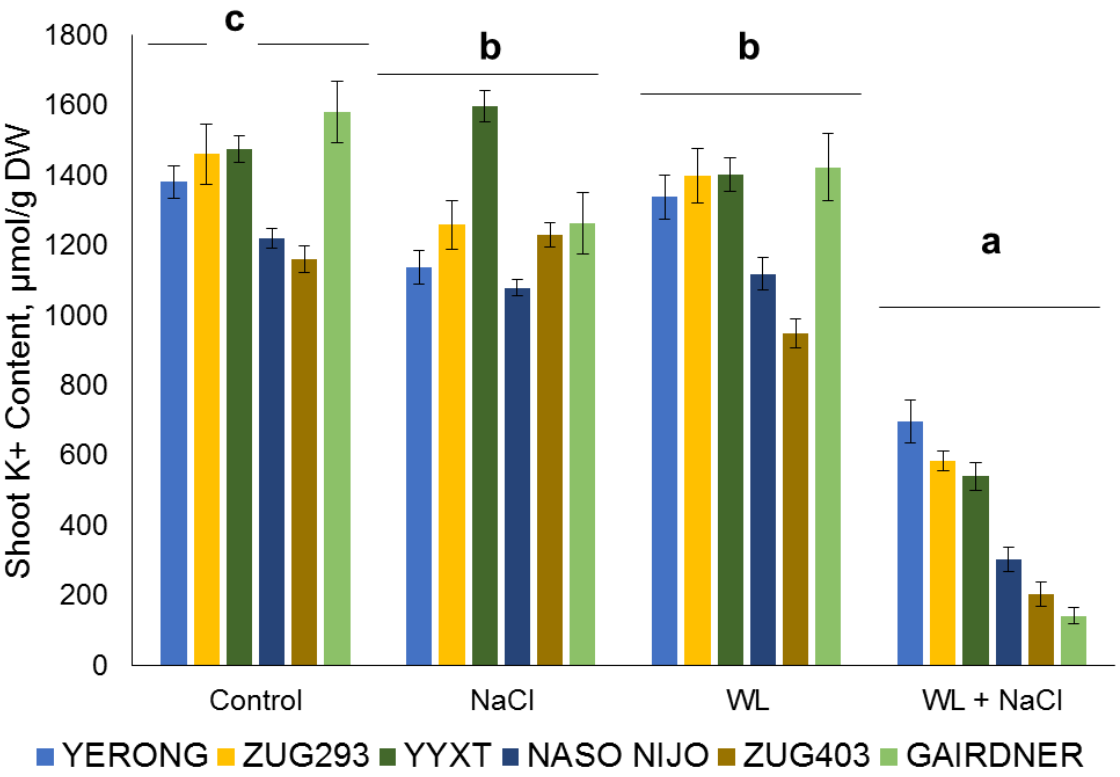

Figure 4.19. Effects of separate and combined salinity and waterlogging stresses on tissue K+ content of selected 6 barley varieties. Measurements were taken 15 days after the treatment onset. For growth conditions and details of treatments refer to Material and Methods section. Different lower case letters indicate the significant difference between treatments (averaged for all 6 varieties) at P< 0.01, the error bars indicate the standard error of all replicated for each treatment/variety

Table 4.9. The maximum and minimum shoot Na+ and K+ content of selected 6 barley varieties under separate and combined stresses of NaCl and WL relative to the control (%)

	Na^+ Content	**K^+ Content**
NaCl relative to control	1118 – 2355 %	80 – 108 %
WL relative to control	133 – 299 %	82 – 97 %
WL/NaCl relative to control	2074 – 6595 %	9 – 50 %

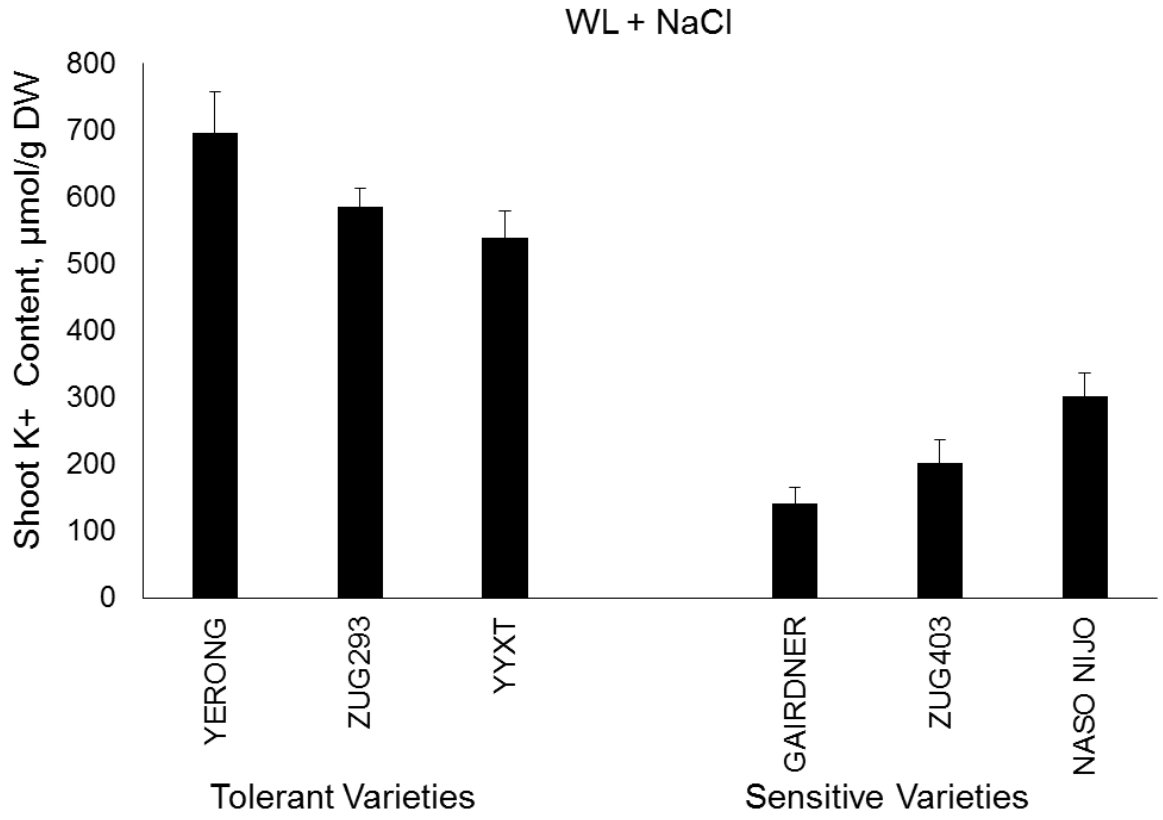

Figure 4.20. Effects of separate and combined salinity and waterlogging stresses on tissue K+ content of selected 6 barley varieties divided in two groups of sensitive and tolerant to combined stresses of salinity and waterlogging. Measurements were taken 15 days after the treatment onset. For growth conditions and details of treatments refer to Material and Methods section. the error bars indicate the standard error of all replicated for each treatment/variety

Comparing the Na^+ and K^+ content of control and treated plants (with separate and combined stresses of NaCl and WL) correlation to the Lit damage index showed that plants under WL conditions had the highest positive correlation for Na^+ content and highest negative correlation for K^+ content with damage index compared to control and two other treatments. WL/NaCl did not show a good correlation between Lit damage index and K^+ content (Figure 4.21).

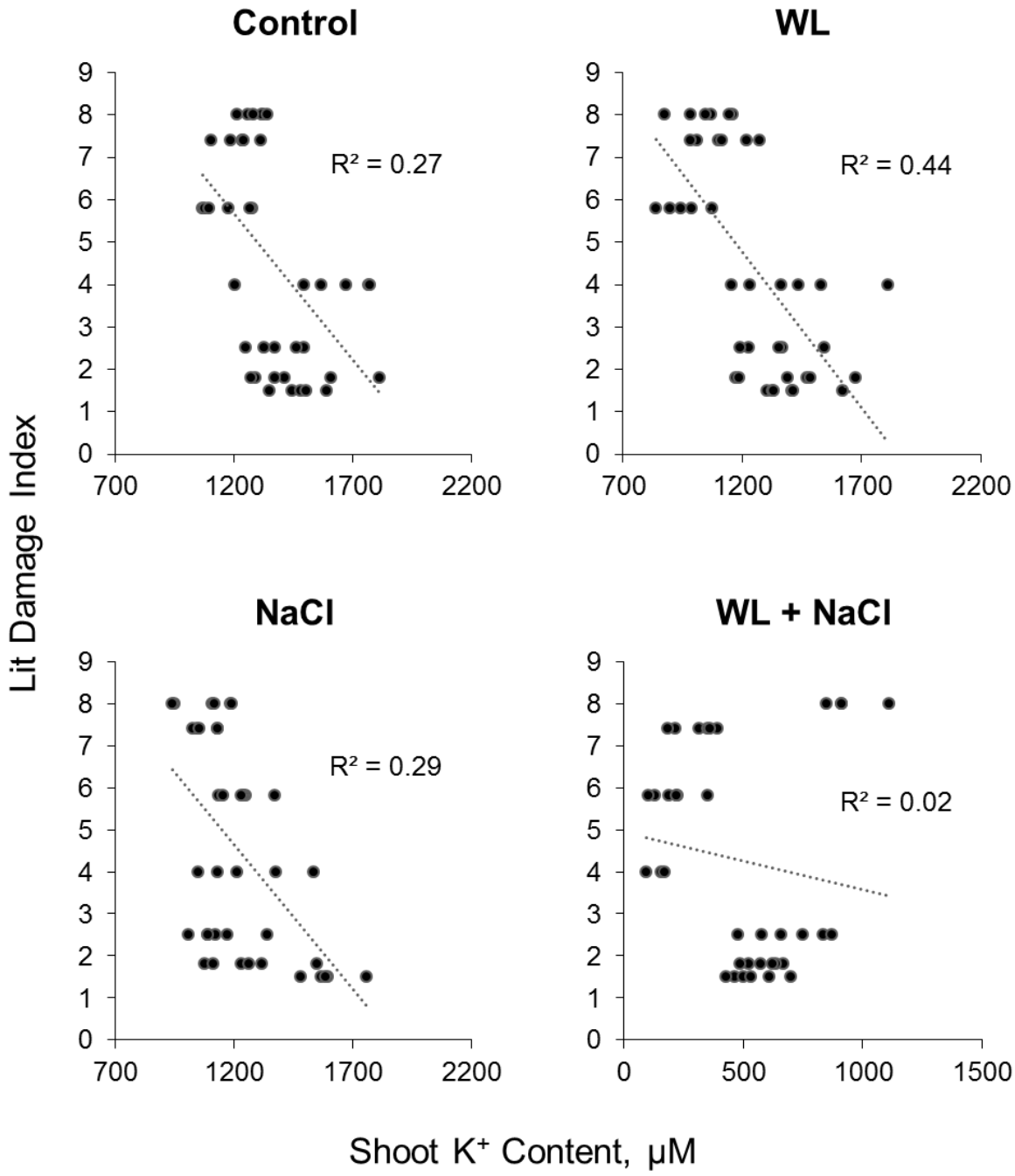

Figure 4.21. Correlation between shoot K^+ content and damage index of barley varieties grown under separate and combined stresses of salinity and waterlogging and control conditions

4.3 Discussion

The adverse effects of combined WL/NaCl stress is more severe than either salinity or waterlogging stress and the combined effects are synergistic but not additive

The current study showed that WL/NaCl stress is more severe than each of the separate stresses and their effect is synergistic but not additive (Figure 4.3). This may be due to the fact that the capacity for aerenchyma induction is reduced under saline conditions (Naidoo and Mundree 1993). Plants respond to waterlogging *per se* by mechanisms such as: root oxygen demand reduction and internal ventilation increase. When waterlogging is combined with salinities higher than 100 mmol, these adaptive anatomical and morphological responses are not adequate to maintain aerobic respiration which is indicated by a significant increase in roots ADH (alcohol dehydrogenase) activity (Naidoo and Mundree 1993). The 250mM NaCl applied to the plants under WL conditions in the current study explains the severe effects of WL/NaCl stress in terms of shoot biomass (Figure 4.4), chlorophyll content (Figure 4.6), chlorophyll fluorescence (Figure 4.10), Na^+ and K^+ content (Figures 4.14 and 4.17).

This study confirms the view expressed earlier (Barrett-Lennard and Shabala 2013), WL/NaCl stress leads to higher concentrations of Na^+ and lower concentrations of K^+ compared to NaCl stress under drained conditions (Figure 4.14). Results from the current study also showed barley varieties treated with WL/NaCl have a much more dramatic increase in Na^+ and decrease in K^+ compared to the same varieties under non-saline waterlogged conditions Figure 4.17). One of the major factors of a plants tolerance to salinity is the intracellular K^+/Na^+ homeostasis (Maathuis and Amtmann (1999), Shabala and Cuin (2008)). There might not be a clear explanation for the cellular and molecular mechanisms of increased Na^+ and decreased K^+ concentrations under WL/NaCl stress but possible elucidations are as follows:

First, a common transport system could conceivably be in charge for simultaneous but oppositely directed changes in Na^+ and K^+ in plants affected by WL. On the other hand, in saline conditions, NSCC (non-selective cation channels), that are known to be virtually just as permeable to both Na^+ and K^+ (Demidchik and Tester 2002), are introduced for their Na^+ entry into the roots in most species (Demidchik and Maathuis 2007). Alongside this an enormous K^+ leakage may happen from the plant root as a result of NSCC channel activation

by ROS (reactive oxygen species) production (Demidchik and Maathuis 2007) under WL/NaCl stress.

Second, the plasma membrane SOS1 Na^+/H^+ antiporters that dynamically expel Na^+ from the cytosol (Shi et al. 2002) and third, depolarization-activated outward-rectifying (GORK) (in Arabidopsis; Ache et al. (2000)) channels that are responsible for K+ retaining in the cytosol play the crucial role in intracellular K^+/Na^+ homeostasis. Both of these transporters require oxygen to function. Oxygen is ordinarily needed in roots for optimum production of adenosine triphosphate (ATP) from sugars. Roots under WL *per se* and WL/NaCl conditions use the oxygen to the point at which the oxygen shortage restricts aerobic respiration. The ensuing anaerobic respiration reduces the ATP production from 30-36 mol ATP via mitochondrial oxidative phosphorylation in drained conditions to 2–4 mol ATP via glycolysis per hexose (Bailey-Serres and Voesenek 2008). The reduction in ATP concentration in waterlogged roots will undermine a plants' capability to fuel H^+-ATPase, with effects to both Na^+ exclusion and K^+ retention (Zeng et al. 2013). First, the SOS1 antiporters operation is depended on sharp H^+ gradients through the plasma membrane which is powered by the plasma membrane H^+-ATPase activity (Palmgren and Harper 1999). Second, the H^+-pump is a vital electrogenic factor to provide sufficient negative membrane potential while keeping GORK channels shut when membrane is extensively depolarized due to salinity. Salinity even in drained situation leads to extensive membrane depolarization (by 60–80 mV;(Shabala and Cuin 2008)).

Tolerance to WL/NaCl stress is determined mostly by sensitivity to WL

In general, joint WL and NaCl stresses produced a combination of the effects of each stress applied independently, even though the current study showed that WL had a greater contribution in limiting factors compared to salinity. Plant response to WL/NaCl showed higher significant correlation with WL compared to NaCl in several measured morphological and physiological characteristics such as FW and DW (Table 4.2, Figure 4.7), chlorophyll content SPAD value (Table 4.6) and maximum photochemical efficiency of PSII (Chlorophyll Fluorescence Fv/Fm Value) (Table 4.8). Data from several sources have identified that plant reactions to NaCl and WL stresses have much in common (Munns 2002). Plants function through a multipart network of signal transduction pathways to tolerate the salt and hypoxic stresses. As explained above, oxygen is a necessary component for producing ATP. Therefore, changing the root environment from drained to waterlogged leads

to about a 95% reduction in ATP production and plant tissues precede to a state of energy crisis. As ATP is the fuel for almost all cellular processes during anaerobic conditions plant cells need to minimize their energy requirements for maintenance and spend most of their energy for critical functions to survive (Gibbs and Greenway 2003). Energy requirements to maintain the plants functions under each NaCl and WL stress are discussed below.

Plants make several structural adjustments for adaption to anaerobic conditions such as lysigenous aerenchyma in order to assist with gas exchange between aerial and flooded organs (Bailey-Serres and Voesenek 2008). Plants need to spend some energy to organise programmed death for specific cortical and epidermal cells to create lysigenous aerenchyma. This programmed death may be activated by submergence-induced ethylene production followed by localized accumulation of ROS (Steffens and Sauter 2009; Parlanti et al. 2011; Steffens et al. 2011). It was discovered that spatial regulation of epidermal cell death requires the mechanical force that is produced through ethylene and ROS-mediated adventitious root growth in some plants such as rice (Steffens et al. 2012).

K^+ but not Na^+ ionic relations explains the tolerance to WL/NaCl stress

Potassium is a key cation in the cytosol of plants as a counter ion to equilibrate the negatively charged proteins and nucleic acids and is crucial for enzyme activation, protein synthesis stabilisation, membrane potential development, maintenance of turgor pressure and cytosolic pH homeostasis and facilitating all forms of plant activities (Shabala 2003; Dreyer and Uozumi 2011; Anschütz et al. 2014; Chérel et al. 2014). High Na^+ content in plants due to exposing to saline conditions can cause a competition between Na^+ and K^+ because of the physiochemical similarities of Na^+ with K^+ (Tester and Davenport 2003; Chen et al. 2008; Cuin et al. 2008).

Saline conditions under hypoxia, with few exceptions, causes an increase in Na^+ and Cl^- concentration and decrease in K^+ concentration, compared to drained saline conditions (Barrett-Lennard and Shabala 2013). It is shown that not only Na^+ and Cl^- increase but also K^+ reduction affect the plant growth negatively under WL/NaCl stress (Barrett-Lennard 2003) even though the current study confirms the greater role of K^+ compared to Na^+ to explain the tolerance to WL/NaCl stress. The previous study in our lab shows that salinity tolerance correlates with K^+ retention but not Na^+ exclusion from the shoot. All barley genotypes showed reduction in K^+ content under saline conditions and the larger the vacuolar K^+ pool was, the lower the resultant leaf injury index (Zhu et al. 2015)

Thus far, the previous studies suggested that WL affects membrane selectivity and nutrient transport (Morard and Silvestre 1996; Drew 1983; Buwalda et al. 1988a) as well as small molecule weight metabolites and ion maintenance by roots (Buwalda et al. 1988b). It is hypothesized that two types of damage can happen to membrane barriers or processes during waterlogging. Firstly, considerable damage to membrane integrity results in the uptake and transport of ions by mass flow which explains the decrease of phosphate, potassium, calcium and magnesium concentration in wheat's xylem. Secondly, insufficient energy (ATP) inhibits ion influx and efflux (Barrett-Lennard 2003).

In summary, barley plants response to combined WL/NaCl stress is mostly affected by the waterlogging although its effects are much more severe than waterlogging *per se* stress on plants. Furthermore, K^+ reduction in the plants under combined WL/NaCl stress was found to be more critical compared to Na^+ increase.

Chapter 5: Effects of WL and salinity stresses and their combination on shoot and root agronomical and physiological characteristics of hydroponic-grown plants

5.1 Introduction

Many mechanisms conferring plant tolerance to both salinity and waterlogging stresses are located in the roots. For instance, under saline conditions, the root is the first "checkpoint" for Na^+ removal (Shabala 2012). Na^+ exclusion from uptake is engaged with channels and transporters such as NSCC, HKT and LCT (Su et al. 2015; Demidchik and Maathuis 2007; Amtmann et al. 2001). Furthermore, control of Na^+ xylem loading from the roots plays a major role amongst various tolerance physiological mechanisms in the plant (Munns and Tester 2008). There was a strong correlation between root K^+ retention and plant salinity tolerance under saline conditions (Chen et al. 2007b). Under waterlogging conditions also, there are a number of tolerance mechanisms that are engaged within the root such as aerenchyma formation, oxygen transport from the roots, control of radial oxygen loss and anaerobic metabolism of the roots (See Section 2.5 in Chapter 2). Therefor root function under combined stresses needs to be studied.

After the whole shoot study of the barley plants under salinity and waterlogging stresses in soil, hydroponic experiments were designed to monitor and compare the changes of the root biomass and its physiological characteristics under separate and combined salinity and waterlogging stresses. Easy access to the roots under hydroponic experiments was not the only aim of this study but also the fact that plants grown under hydronic conditions are exposed to more uniform conditions, with no radial and longitudinal gradients in the rhizosphere. To simulate the effects of waterlogging N_2 was bubbled through the agar nutrient solution to apply WL and WL/NaCl stresses to the plants. However, it should be noted that there can be significant differences between N_2-bubbled agar nutrient solutions and waterlogged soils. Oxygen concentrations of the agar nutrient solution can decrease to 0.003 mol m^{-3} (1% of the concentration in the aerated non-agar nutrient solutions in control and

saline treatments) in a few minutes of N_2-bubbling, while such decrease in O_2 concentrations in waterlogged soils generally takes several days (Barrett-Lennard 1986).

5.2 Results

5.2.1 Shoot Growth Performance

Shoot growth performance was assessed by measuring three main characteristics including fresh weight, dry weight and length.

5.2.1.1 Shoot Fresh Weight

Plant shoot fresh weight (FW) was significantly ($P<0.01$) reduced by separate and combined NaCl and WL stress after 8 and 16 days. Shoot FW was decreased by 1.3 to 2.4, 1.6 to 2.2 and 2.0 to 3.1 fold respectively under NaCl, WL and WL/NaCl stresses after 8 days of stress while it was decreased by 1 to 4, 2 to 5 and 5 to 16 fold respectively after 16 days of stress (Figure 5.1). Plant FW had more reduction after 16 days stress compared to 8 days for all three stresses while WL/NaCl had the most effect on the shoot FW (Figure 5.1).

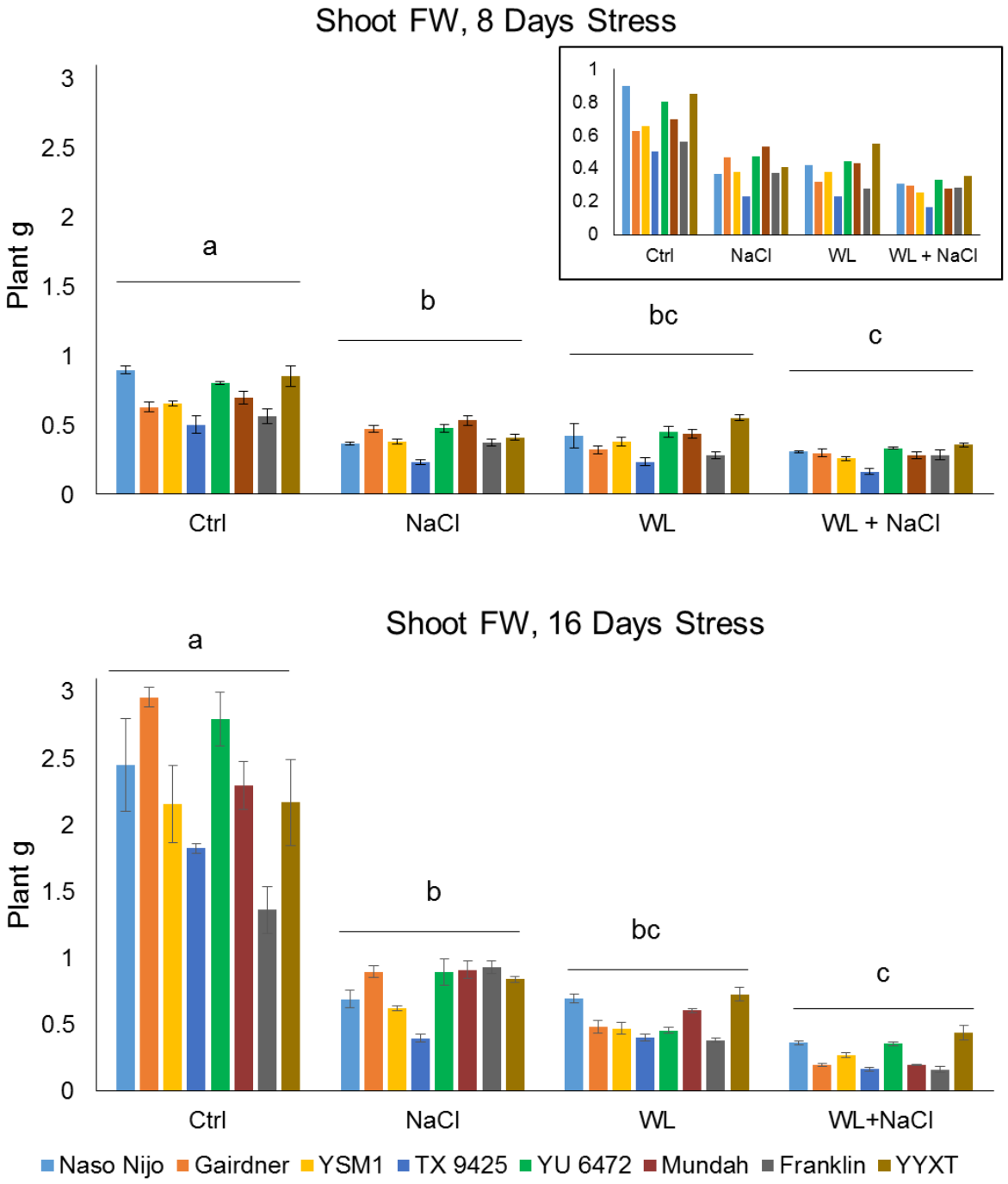

Figure 5.1. Effects of separate and combined stresses of salinity and waterlogging on shoot fresh weight of selected 8 barley varieties under hydroponic conditions. 6 day old seedlings were subjected to one of the four treatments; Control (No NaCL, well drained), NaCl (irrigated by 150mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 150mM NaCL solution). Plants were harvested after 8 and 16 days stress for biomass measurements. The lower case letters indicate the significant difference between treatments (averaged for all 8 varieties at $P<0.01$), the error bars indicate the standard error of all replicated for each treatment/variety

The average shoot FW under NaCl stress was 41% to 76% relative to control with Naso Njio and Mundah after 8 days while it was 22% to 68% with TX9425 and Franklin after 16 days for the most and least reduction, respectively. The average shoot FW after 8 days WL stress ranged from 46% to 64%, TX9425 and YYXT had the most and least reduction, respectively. The average shoot FW was 16% to 34% withYU6472 and Gairdner for the most and YYXT for the least reduction. The average shoot FW under WL/NaCl stress was 33% to 50% with TX9425 and Franklin after 8 days and it was 7% to 20% with Gairdner and YYXT for the most and least reduction, respectively.

Table 5.1. The average shoot fresh weight (FW) of selected 8 barley varieties relative to control (%) after 8 and 16 days of separate and combined NaCl and WL stresses

	Cultivar/ Treatment	FW (% Control), 8 days Stress			FW (% Control), 16 Days Stress		
		NaCl	WL	WL/NaCl	NaCl	WL	WL/NaCl
Shoot	Naso Nijo	41	55	34	28	28	15
	Gairdner	75	51	47	30	16	7
	YSM1	57	58	39	29	22	12
	TX9425	46	46	33	22	22	9
	YU6472	59	56	41	32	16	13
	Mundah	76	62	40	40	26	8
	Franklin	66	50	50	68	28	12
	YYXT	48	64	42	39	34	20

The results from the statistical analysis of FW reduction under all three applied stresses after 8 and 16 days of treatment are summarised in Table 5.2 and Figure 5.2. Plant FW after 8 days NaCl stress showed stronger correlation with plant FW under WL/NaCl stress compared to WL stressed plants. While plant FW after 16 days WL/NaCl stress was more correlated with WL stressed plants compared to NaCl (Figure 5.2). In other words, average FW of all barley varieties under WL/NaCl is more correlated to NaCl after 8 days stress and it is more correlated to WL after 16 days stress (Figure 5.2).

The correlation between the effects of 8 and 16 days NaCl stress on shoot FW was more than WL stress, while the correlation between the effects of 8 and 16 days WL/NaCl stress was less than either NaCl or WL stress (Table 5.2).

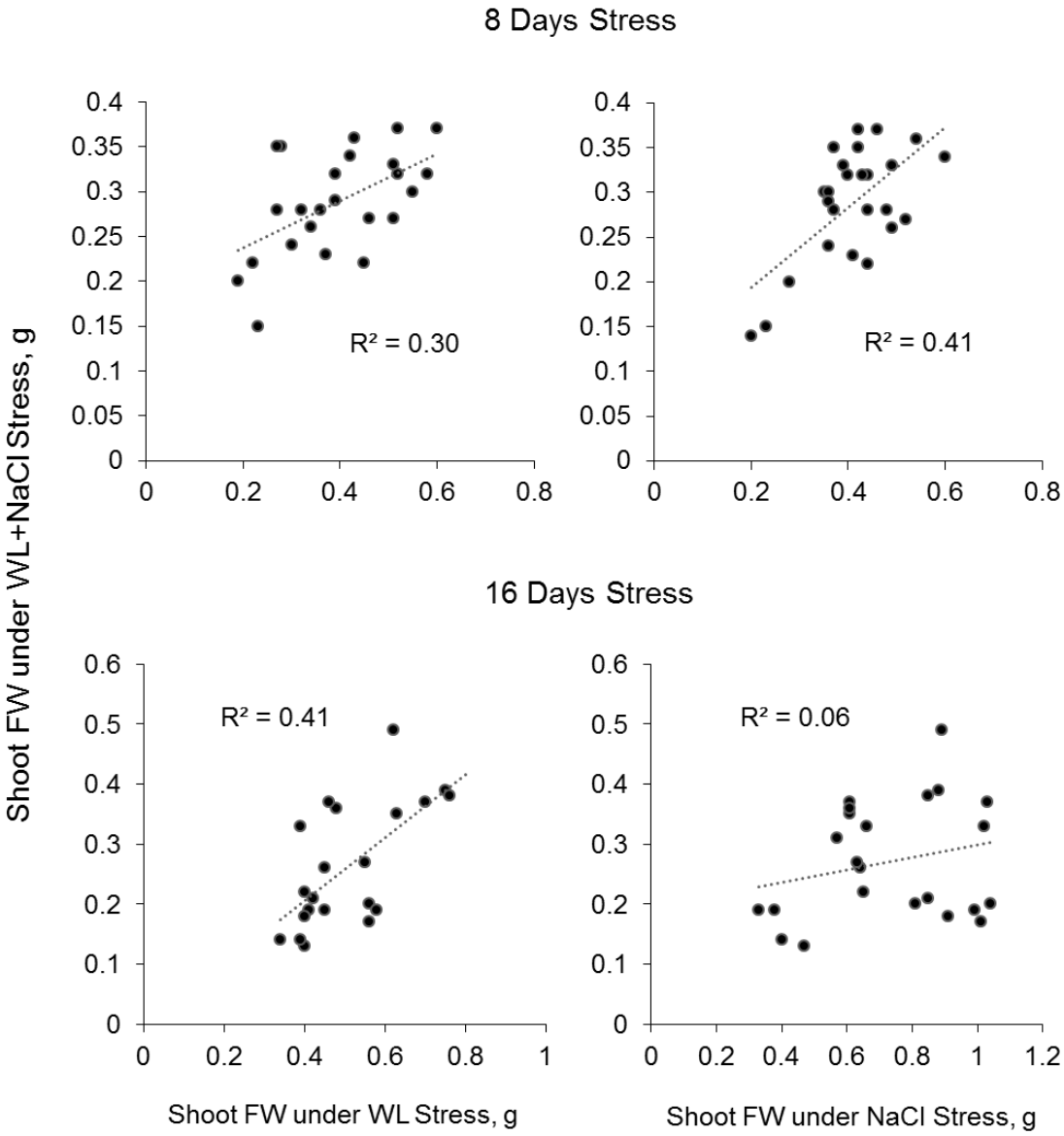

Figure 5.2. Correlation between separate NaCl and WL stress and their combination after 8 and 16 days stress

Table 5.2. The correlation between the effects of 8 and 16 days separate NaCl and WL stress and their combination on shoot FW

	Fresh Weight, 8 days treatment		
	NaCl	**WL**	**WL/NaCl**
FW, 16 days stress, NaCl	0.750**		
FW, 16 days stress, WL		0.706**	
FW, 16 days stress, WL/NaCl			0.597**

5.2.1.2 Shoot Dry Weight

16 days of separate and combined NaCl and WL stresses had more effect on shoot DW compared to 8 days stress. The shoot DW reduction after 8 days stress was not significantly different between the treatments while they all showed significant DW reduction compared to control plants. The shoot DW of barley plants under 16 days of separate NaCl and WL stresses and their combination was significantly decreased compared to control plants. The difference between the effects of WL and WL/NaCl on shoot DW after 16 days stress was not significant while both showed more DW reduction compared to NaCl (Figure 5.3).

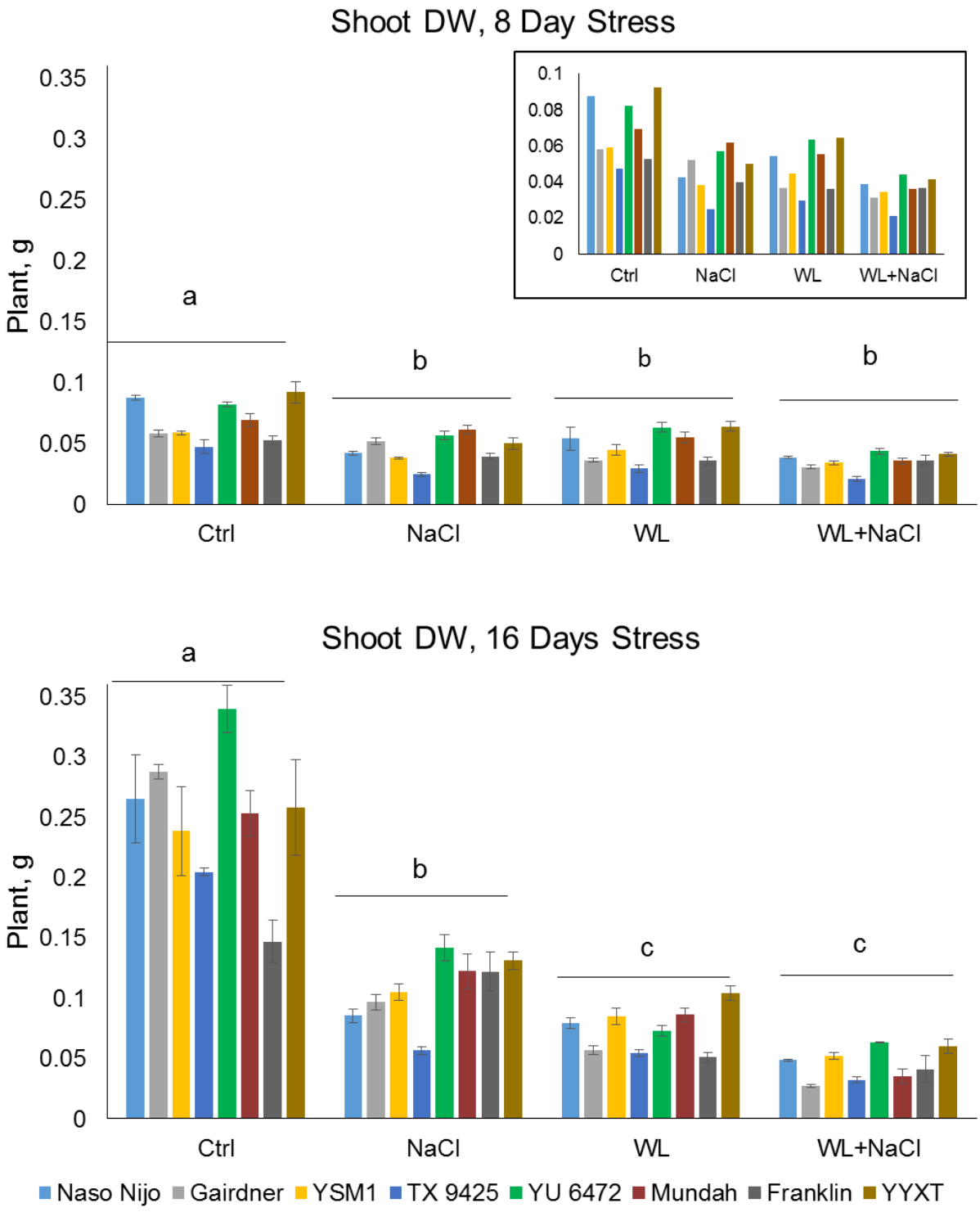

Figure 5.3. Effects of separate and combined stresses of salinity and waterlogging on shoot dry weight of selected 8 barley varieties under hydroponic conditions. 6 day old seedlings were subjected to one of the four treatments; Control (No NaCL, well drained), NaCl (irrigated by 150mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 150mM NaCL solution). Plants were harvested after 8 and 16 days stress for biomass measurements. The lower case letters indicate the significant difference between treatments (averaged for all 8 varieties at $P<0.01$), the error bars indicate the standard error of all replicated for each treatment/variety

The average shoot DW after 8 days NaCl stress, reduced to 48% to 89% relative to control, Naso Nijo had the most and Gairdner and Mundah had the least reduction. All eight varieties under the same stress after 16 days of treatment had 28% to 83% of dry weight relative to the control, TX9425 and Franklin had the most and least reduction, respectively. WL stress after 8 days reduced the dry weight of the plants from 63% to 80% relative to control, the greatest reduction was from Gairdner and TX9425 while the least was from Mundah. Barley plant shoot DW under the same stress after 16 days reduced by 20% to 40% relative to control, Gairdner and YYXT had the greatest and least reduction respectively. The average shoot DW under WL/NaCl stress after 8 days ranged from 44% to 69% relative to the control, Naso Nijo and TX9425 had the most and Franklin had the least reduction after 8 days. The average shoot DW was 9% to 28% after 16 days and Gairdner and Franklin had the most and least reduction, respectively.

Table 5.3. The average shoot dry weight (DW) of selected 8 barley varieties relative to control (%) after 8 and 16 days of separate and combined NaCl and WL stresses

	Cultivar/ Treatment	DW (% Control), 8 Days Stress			DW (% Control), 16 Days Stress		
		NaCl	WL	WL/NaCl	NaCl	WL	WL/NaCl
Shoot	Naso Nijo	48	70	44	32	30	18
	Gairdner	89	63	53	34	20	9
	YSM1	65	76	58	44	36	22
	TX9425	52	63	44	28	27	16
	YU6472	69	77	53	42	21	19
	Mundah	89	80	52	48	34	14
	Franklin	75	68	69	83	35	28
	YYXT	54	70	45	51	40	23

5.2.1.3 Shoot Length

8 and 16 days NaCl, WL and WL/NaCl stresses reduced shoot length of all selected barley plants. The effects of 8 days NaCl, WL and WL/NaCl stresses on shoot length was not

significant between treatments while they all showed a significant reduction compared to control plants. WL/NaCl stress had the most effect on plant shoot length after 16 days followed by WL and then NaCl, shoot length of all stressed plants was significantly less than control plants (Figure 5.4).

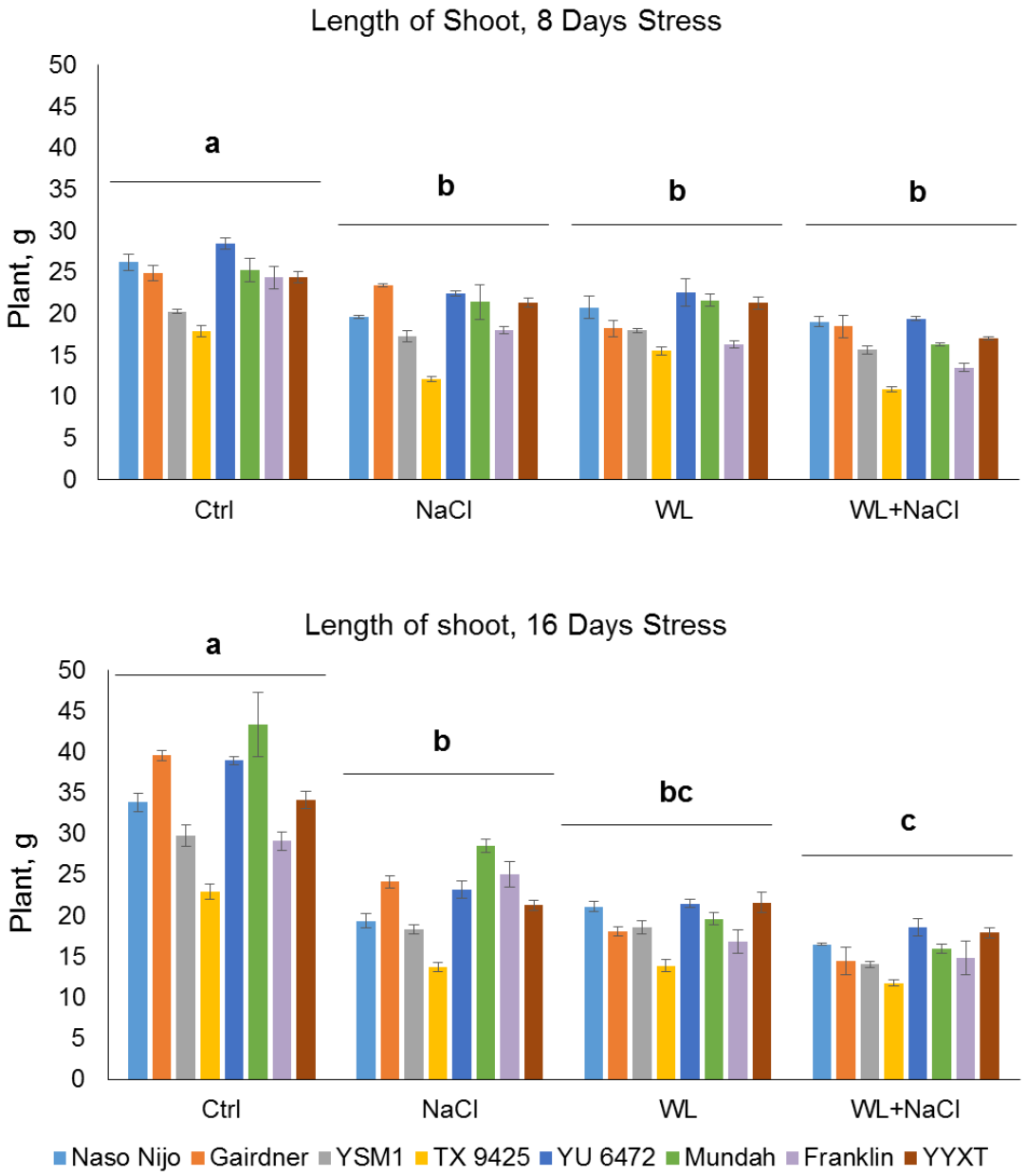

Figure 5.4. Effects of separate and combined stresses of salinity and waterlogging on shoot length of selected 8 barley varieties under hydroponic conditions. 6 day old seedlings were subjected to one of the four treatments; Control (No NaCL, well drained), NaCl (irrigated by 150mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 150mM NaCL solution). Plants were harvested after 8 and 16 days stress for biomass measurements. The lower case letters indicate the significant difference between treatments (averaged for all 8 varieties at $P<0.01$), the error bars indicate the standard error of all replicated for each treatment/variety

Plants shoot length after 8 days NaCl stress was 68% to 94% relative to control for TX9425 and Gairdner, and after 16 days was 57% to 86% for Naso Nijo and Franklin, respectively. Franking showed the most reduction in shoot length compared to other varieties after 8 days WL and WL/NaCl stress with 67% and 55% shoot length relative to control, respectively. YSM1 showed the least reduction in shoot length after 8 days WL and WL/NaCl stress with 89% and 77%, respectively. Mundah and Gairdner with 45% and 37% shoot length relative to control showed the most reduction under 16 days of WL and WL/NaCl stress, respectively. While YYXT showed the least reduction under 16 days WL and WL/NaCl stress with 63% and 53% shoot length relative to control, respectively (Table 5.4).

Table 5.4. The average shoot length of selected 8 barley varieties relative to control (%) after 8 and 16 days of separate and combined NaCl and WL stresses

	8 Days Stress	**16 Days Stress**
NaCl relative to control	68 – 94%	57 - 86%
WL relative to control	67 – 89%	45 – 63%
WL/NaCl relative to control	55 – 77%	37 – 53%

5.2.2 Root Growth Performance

5.2.2.1 Root Fresh Weight

8 and 16 days NaCl, WL and WL/NaCl stresses reduced the root FW of all selected barley plants compared to control. The effects of 8 days WL and WL/NaCl stresses on root FW was not significant between treatments while they both showed a significant reduction compared to NaCl plants. WL/NaCl stress had the most effect on plant root FW after 16 days followed by WL and then NaCl, root FW of all stressed plants was significantly less than control (Figure 5.5).

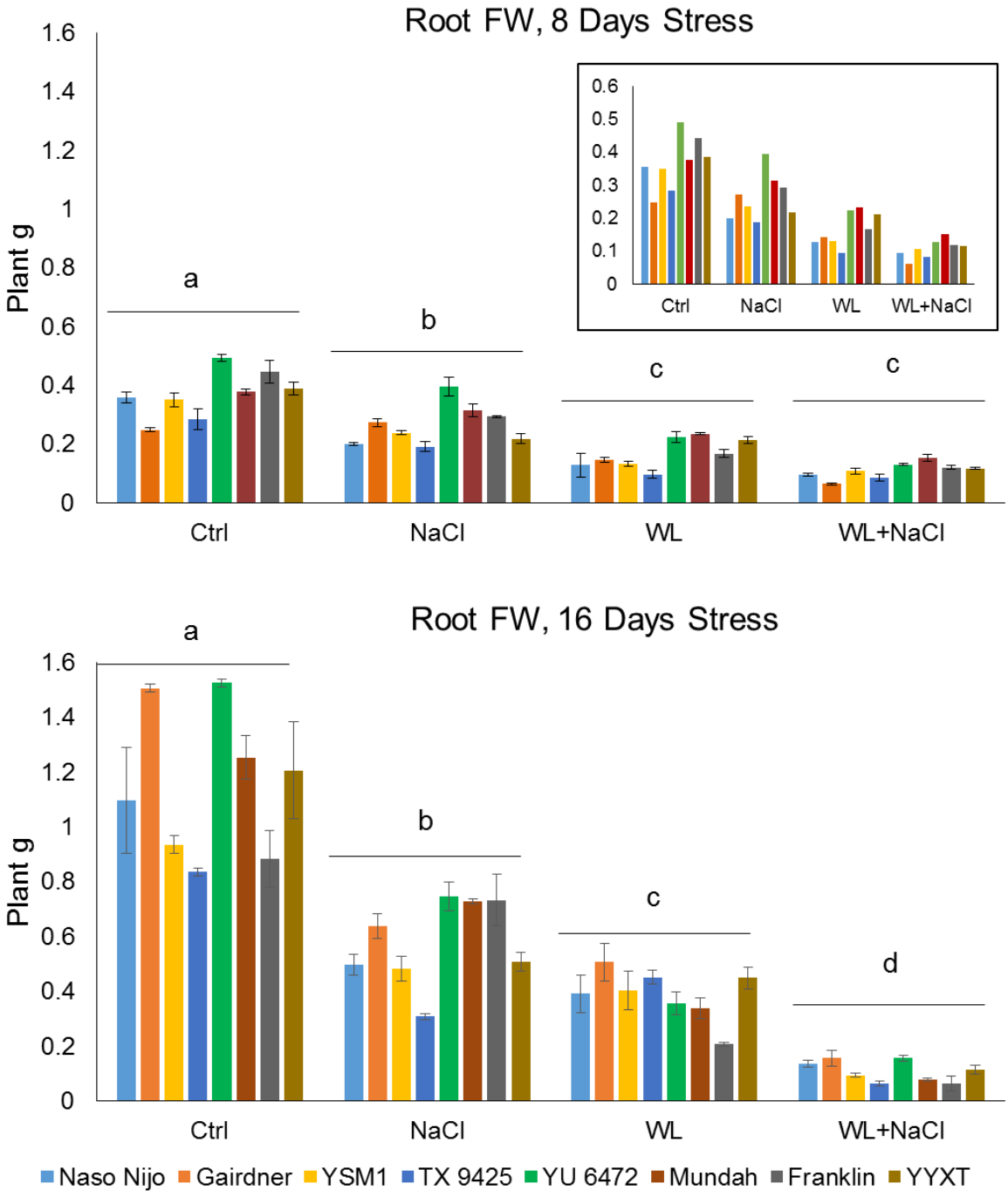

Figure 5.5. Effects of separate and combined stresses of salinity and waterlogging on root fresh weight of selected 8 barley varieties under hydroponic conditions. 6 day old seedlings were subjected to one of the four treatments; Control (No NaCL, well drained), NaCl (irrigated by 150mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 150mM NaCL solution). Plants were harvested after 8 and 16 days stress for biomass measurements. The lower case letters indicate the significant difference between treatments (averaged for all 8 varieties at $P<0.01$), the error bars indicate the standard error of all replicated for each treatment/variety

The average root FW after 8 days NaCl stress was 56% to 110% relative to control, Naso Nijo and YYXT had the most and Gairdner had least reduction. Plants under 16 days of the same treatment showed 37% to 83% FW relative to control, TX9425 and Franklin had the most and least reduction, respectively. The root FW range relative to the control after 8 days WL stress was 34% to 62% for TX9425 and Mundah, respectively. The average root FW after 16 days WL stress was 23% to 54% relative to the control, YU6472 and Franklin had the most and TX9425 had the least reduction. The average FW relative to control after 8 days WL/NaCl stress ranged from 26% to 40%, Gairdner and YU6472 had the most and Mundah had the least reduction. The average root FW under WL/NaCl after 16 days was 6% to 12% relative to control for Mundah and Naso Nijo, respectively (Table 5.5).

Table 5.5. The average root fresh weight (FW) of selected 8 barley varieties relative to control (%) after 8 and 16 days of separate and combined NaCl and WL stresses

	Cultivar/ Treatment	FW (% Control), 8 days Stress			FW (% Control), 16 Days Stress		
		NaCl	WL	WL/NaCl	NaCl	WL	WL/NaCl
Root	Naso Nijo	56	36	27	45	36	12
	Gairdner	110	59	26	42	34	10
	YSM1	68	38	31	52	43	10
	TX9425	67	34	30	37	54	7
	YU6472	80	45	26	49	23	10
	Mundah	84	62	40	58	27	6
	Franklin	66	37	27	83	23	7
	YYXT	56	55	30	42	37	9

There is a stronger positive correlation between shoot and root FW for control plants and plants under NaCl stress compared to plants under WL and WL/NaCl stress (Table 5.6).

Table 5.6. Correlation between the effects of NaCl, WL and WL/NaCl stresses on plant shoot and root fresh weight after 16 days

	Shoot Fresh Weight / 16 days stress			
	Control	**NaCl**	**WL**	**WL/NaCl**
Root FW/ Control	0.848**			
Root FW/ NaCl		0.866**		
Root FW/ WL			0.538**	
Root FW/ WL/NaCl				0.591**

5.2.2.2 Root Dry Weight

8 and 16 days of NaCl, WL and WL/NaCl stress reduced the root DW of all selected barley plants compared to control. The effects of 8 days WL and WL/NaCl stress on root DW was not significant between treatments while they both showed significant reduction compared to NaCl. WL/NaCl stress had the most effect on plant root DW after 16 days followed by WL and NaCl. There was not a significant difference between the effects of NaCl and WL stress after 16 days on barley plants root DW. Root DW of all stressed plants was significantly less than control plants (Figure 5.6).

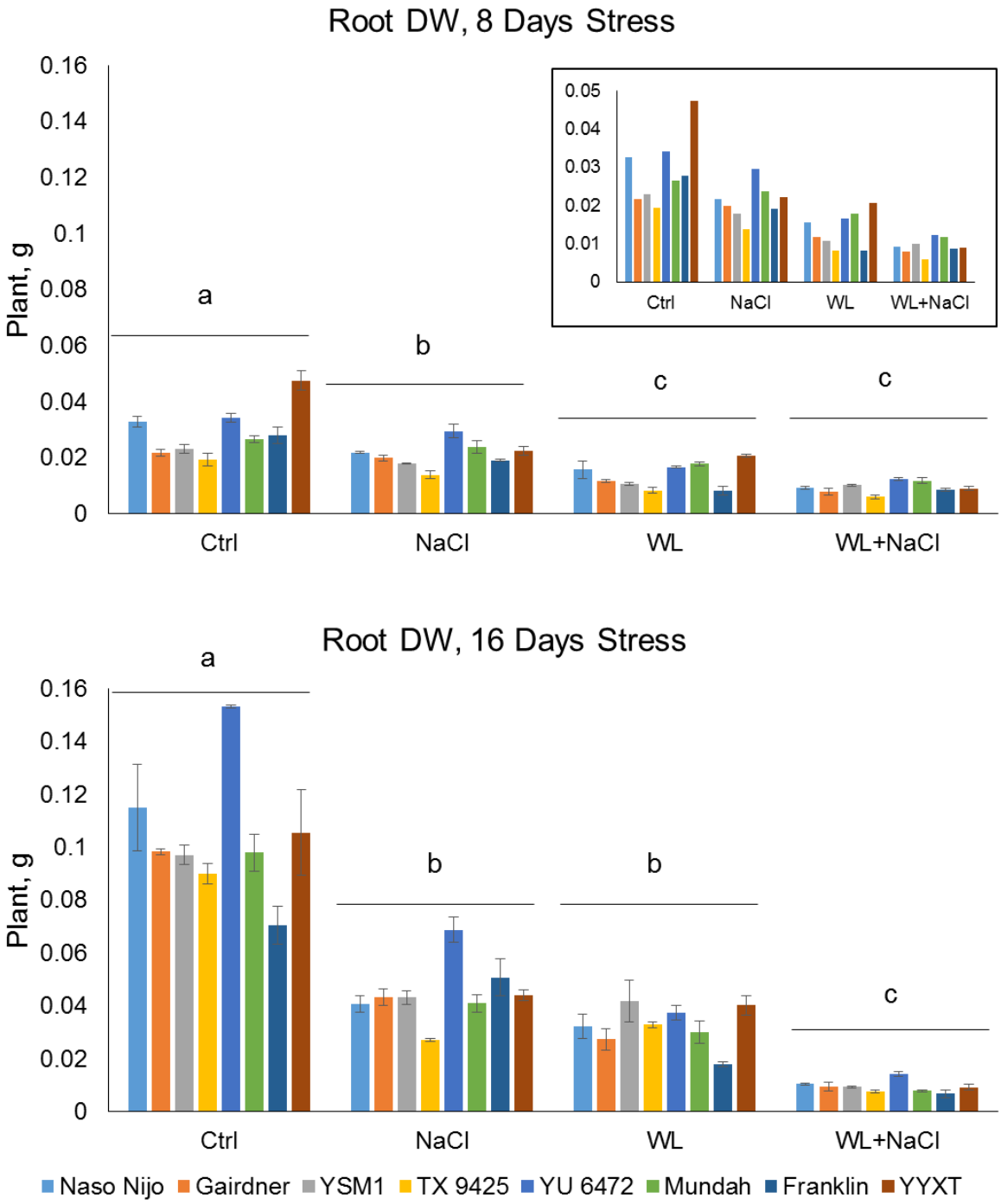

Figure 5.6. Effects of separate and combined stresses of salinity and waterlogging on root dry weight of selected 8 barley varieties under hydroponic conditions. 6 day old seedlings were subjected to one of the four treatments; Control (No NaCL, well drained), NaCl (irrigated by 150mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 150mM NaCL solution). Plants were harvested after 8 and 16 days stress for biomass measurements. The lower case letters indicate the significant difference between treatments (averaged for all 8 varieties at $P<0.01$), the error bars indicate the standard error of all replicated for each treatment/variety

The average root DW after 8 days NaCl stress was 47% to 92% relative to control with YYXT and Gairdner the lowest and highest respectively, while it was 30% to 72% after 16 days with TX9425 and Franklin for the highest and lowest reduction, respectively. The root DW under WL stress relative to control ranged from 29% to 67% for Franklin and Mundah after 8 days; and 24% to 43% for YU6472 and YSM1 after 16 days, respectively. The response rate under WL/NaCl after 8 days stress ranged from 19% to 44% for YYXT with the most reduction from Mundah and the least reduction from YSM1. The average root DW after 16 days WL/NaCl stress was 8% to 10% relative to control; Mundah had the most reduction while Gairdner, YSM1 and Franklin all showed the least reduction (Table 5.7).

Table 5.7. The average root dry weight (DW) of selected 8 barley varieties relative to control (%) after 8 and 16 days of separate and combined NaCl and WL stresses

	Cultivar/ Treatment	DW (% Control), 8 Days Stress			DW (% Control), 16 Days Stress		
		NaCl	WL	WL/NaCl	NaCl	WL	WL/NaCl
Root	Naso Nijo	67	48	28	35	28	9
	Gairdner	92	54	40	44	28	10
	YSM1	78	46	44	44	43	10
	TX9425	71	43	31	30	37	9
	YU6472	86	49	36	45	24	9
	Mundah	89	67	44	42	31	8
	Franklin	68	29	31	72	25	10
	YYXT	47	44	19	42	38	9

5.2.2.3 Root Length

8 and 16 days NaCl, WL and WL/NaCl stresses reduced all selected barley plants root length. The different between effect of 8 days WL and WL/NaCl stresses on root length was not significant between treatments but both showed a reduction compared to NaCl plants. Barley plants root length after 16 days NaCl, WL and WL/NaCl stresses was not significantly different between treatments but showed reduction compared to the control plants (Figure 5.7).

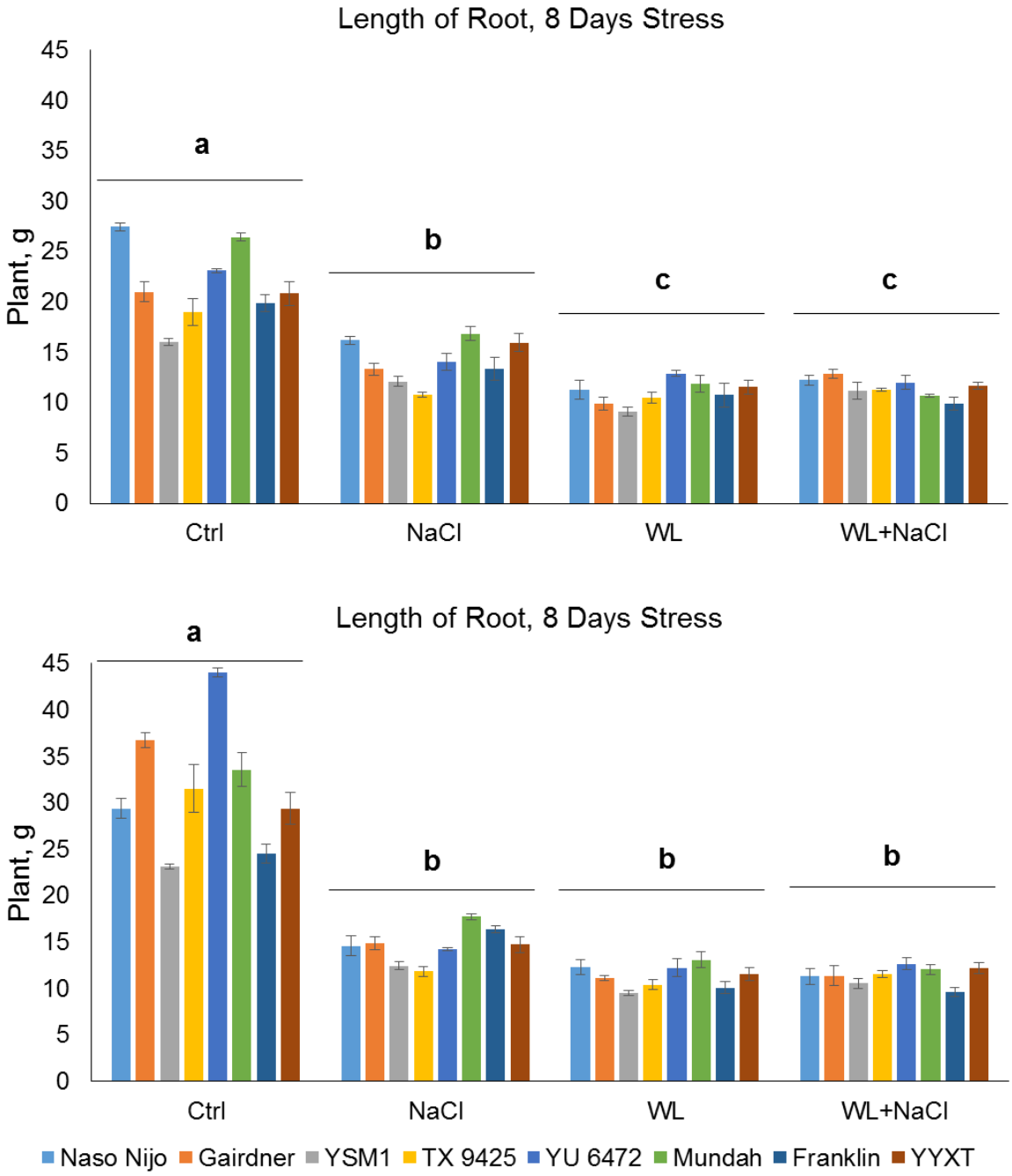

Figure 5.7. Effects of separate and combined stresses of salinity and waterlogging on root length of selected 8 barley varieties under hydroponic conditions. 6 day old seedlings were subjected to one of the four treatments; Control (No NaCl, well drained), NaCl (irrigated by 150mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 150mM NaCl solution). Plants were harvested after 8 and 16 days stress for biomass measurements. The lower case letters indicate the significant difference between treatments (averaged for all 8 varieties at $P<0.01$), the error bars indicate the standard error of all replicated for each treatment/variety

Naso Nijo had the least decrease in length in the first and second 8 day of treatment under WL stress; it had 41% of length relative to control after 8 days and just decreased to 42% after the second 8 days. YU6472 had the most reduction in all three treatments after 16 days with 32%, 28% and 29% for NaCl, WL and WL/NaCl, respectively. YSM1 had the least reduction in the root length after 8 days of treatment under WL and WL/NaCl with 57% and 70% relative to control, respectively.

Table 5.8. The average root length of selected 8 barley varieties relative to control (%) after 8 and 16 days of separate and combined NaCl and WL stresses

	8 days stress	**16 days stress**
NaCl relative to Control	57 – 77%	32 – 67%
WL relative to Control	41 – 57%	28 – 42%
WL/NaCl relative to Control	40 – 70%	29 – 45%

Control plants showed high positive significant correlation between shoot and root length, while it was less for NaCl stressed plants and it reduced to half for WL stressed plants. Plants shoot and root length under WL/NaCl stress showed no significant correlation (Table 5.9 and Figure 5.8).

Table 5.9. Correlation between the effects of 16 days NaCl, WL and WL/NaCl stresses on plant shoot and root length

		Shoot Length, 16 Days Stress			
		Control	**NaCl**	**WL**	**WL/NaCl**
Root Length	**Control**	0.796*			
	NaCl		0.756**		
	WL			0.375*	
	WL/NaCl				0.106

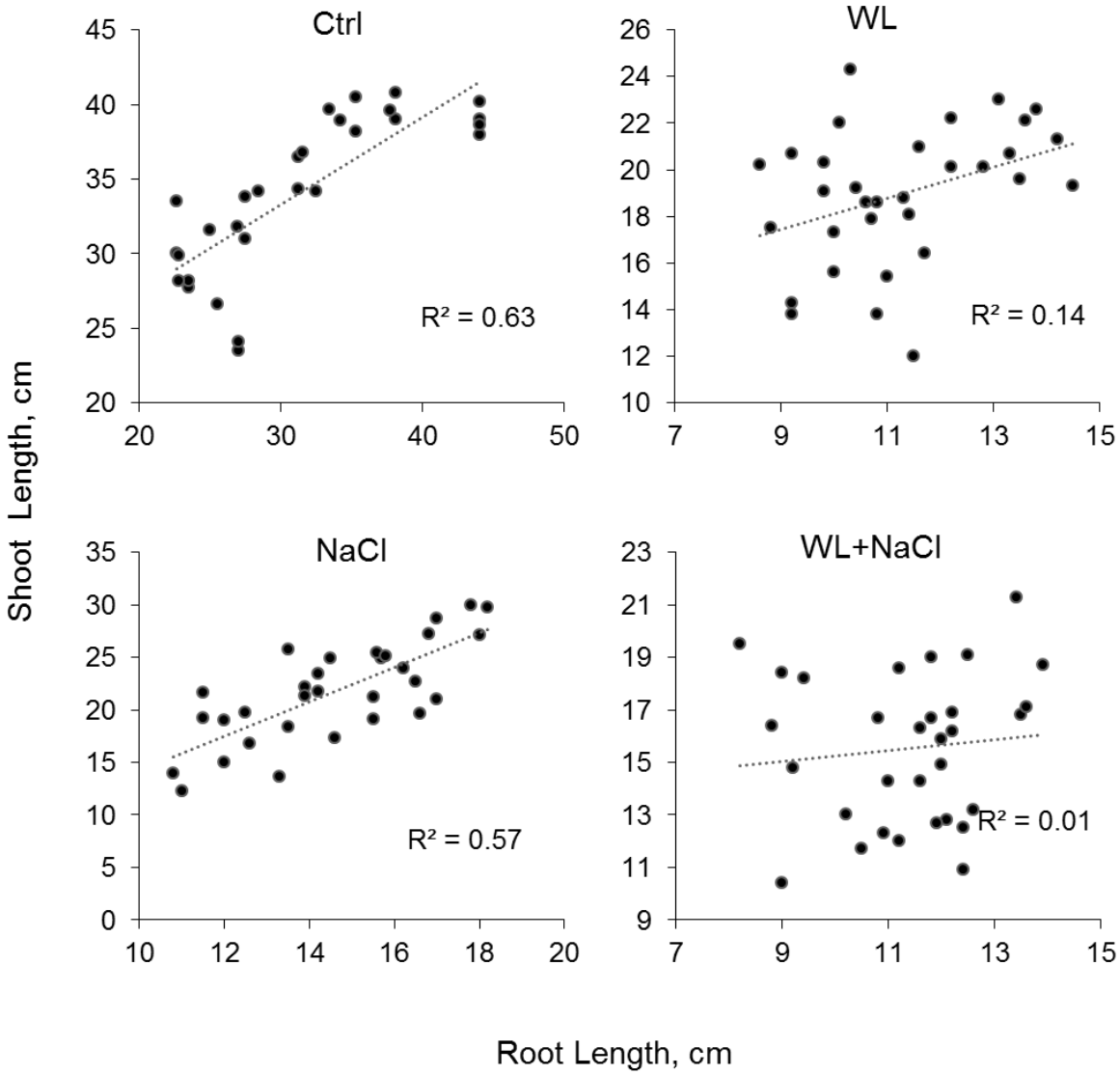

Figure 5.8. The correlation between shoot and root length after 16 days of separate and combined NaCl and WL stress

5.2.3 Chlorophyll Content

Chlorophyll content (Ch C) was significantly reduced by both WL and WL/NaCl stresses after 8 days, while there was no significant difference between control and NaCl treated plants. 16 days of WL/NaCl stress had the most adverse effect on Ch C followed by WL and then NaCl, all treatments were significantly different (Figure 5.9).

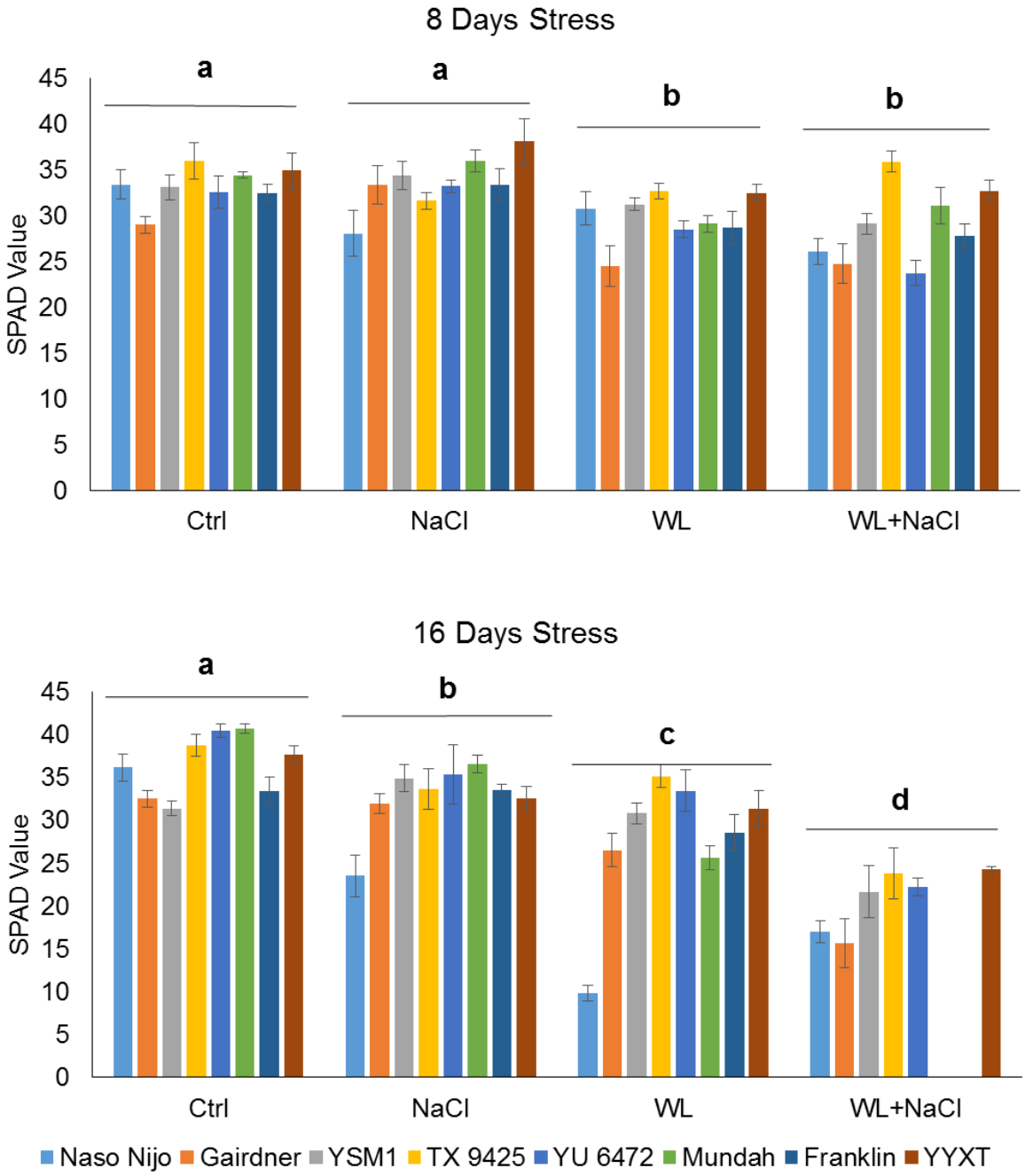

Figure 5.9. Effects of separate and combined stresses of salinity and waterlogging on chlorophyll content SPAD value of selected 8 barley varieties under hydroponic conditions. 6 day old seedlings were subjected to one of the four treatments; Control (No NaCL, well drained), NaCl (irrigated by 150mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 150mM NaCL solution). Plants were measured for SPAD value after 8 and 16 days stress. The lower case letters indicate the significant difference between treatments (averaged for all 8 varieties at $P<0.01$), the error bars indicate the standard error of all replicated for each treatment/variety

Naso Nijo had the least Ch C compared to the rest seven varieties and YYXT had the most after 8 days NaCl stress, while other varieties did not show a signifacant difference. After 16 days of NaCl stress Naso Nijo again showed the least Ch C and the rest of varieties did not have a significant difference (Figure 5.10).

After 8 days of WL stress only Gairdner had a significnatly reduced Ch C. After 16 days WL stress, Naso Nijo had the least Ch C followed by Mundah, Gairdner and Franklin. TX9425 had the most Ch C followed by YU6472 and YYXT (Figure 5.10).

8 and 16 days WL/NaCl stress had more significant effects on chlorophyll content compared to the separate stresses. TX9425 and YYXT in both sampling times were the most tolerant plants. YU9425 showed the lowest Ch C after 8 days but maintained a constant Ch C compared to other varieties in the second eight days and ended up being the third tolerant after YYXT and TX9425. Franklin and Mundah, due to losing the first leaf do not have recorded SPAD while Gairdner and Naso Nijo has the least Ch C after 16 days of treatment (Figure 5.10).

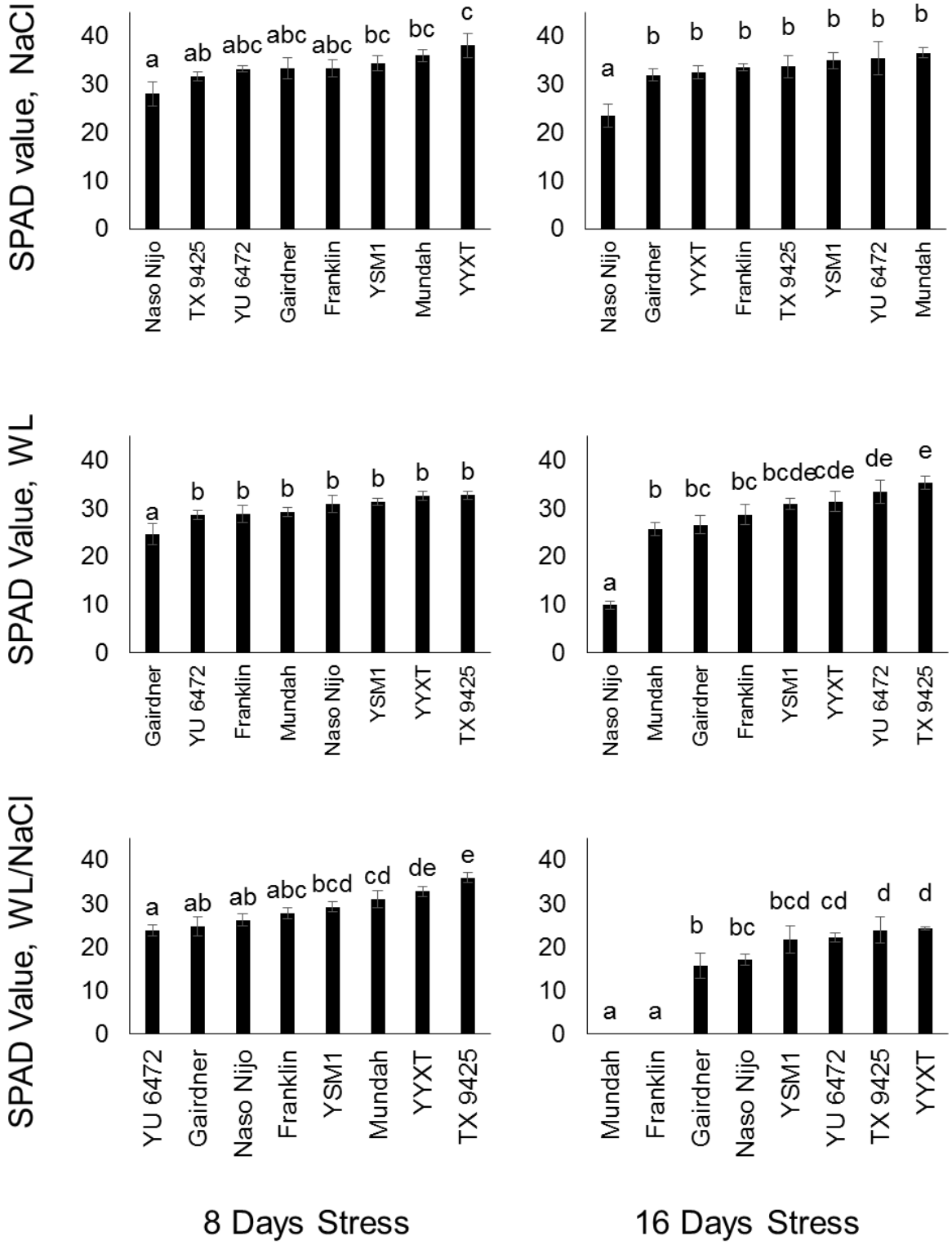

Figure 5.10. The average chlorophyll content, SPAD value of plant under 8 and 16 days NaCl, WL and WL/NaCl stress, analysed statistically by Duncan test

The average chlorophyll content SPAD value after 8 days NaCl stress was 84% to 115% relative to control for Naso Nijo and Gairdner with the most and least reduction respectively. After 16 days of NaCl stress the average SPAD value changed to 65% to 111%, Naso Nijo and YSM1 had the highest and lowest reduction respectively. The average SPAD value under WL stress relative to the control was calculated from 84% to 94% for Gairdner and YSM1 after 8 days; and 27% to 98% for Naso Nijo and YSM1 after 16 days respectively. The average SPAD value under WL/NaCl stresses ranged from 73% to 100% for YU6472 and TX942, after 8 days respectively; and it was 0% for Mundah and Franklin to 69% for YSM1 after 16 days (Table 5.10).

Table 5.10. The average chlorophyll content SPAD value of selected 8 barley varieties relative to control (%) after 8 and 16 days of separate and combined NaCl and WL stresses

Cultivar/ Treatment	Chlorophyl Content, SPAD value (% Control), 8 Days Stress			Chloprophyll Content, SPAD value (% Control), 16 Days Stress		
	NaCl	WL	WL/NaCl	NaCl	WL	WL/NaCl
Naso Nijo	84	92	78	65	27	47
Gairdner	115	84	85	98	82	48
YSM1	104	94	88	111	98	69
TX9425	88	91	100	87	91	62
YU6472	102	88	73	88	83	55
Mundah	105	85	90	90	63	0
Franklin	103	88	86	101	86	0
YYXT	109	93	94	86	83	65

5.2.4 Osmolality

8 days NaCl, WL and WL/NaCl stresses increased all varieties shoot osmolality. WL/NaCl had the most effect on the shoot osmolality followed by NaCl and then WL. 8 days WL stress did not significantly increase the root osmolality and there was not a significant difference between root osmolality of NaCl and WL/NaCl treated plants (Figure 5.11).

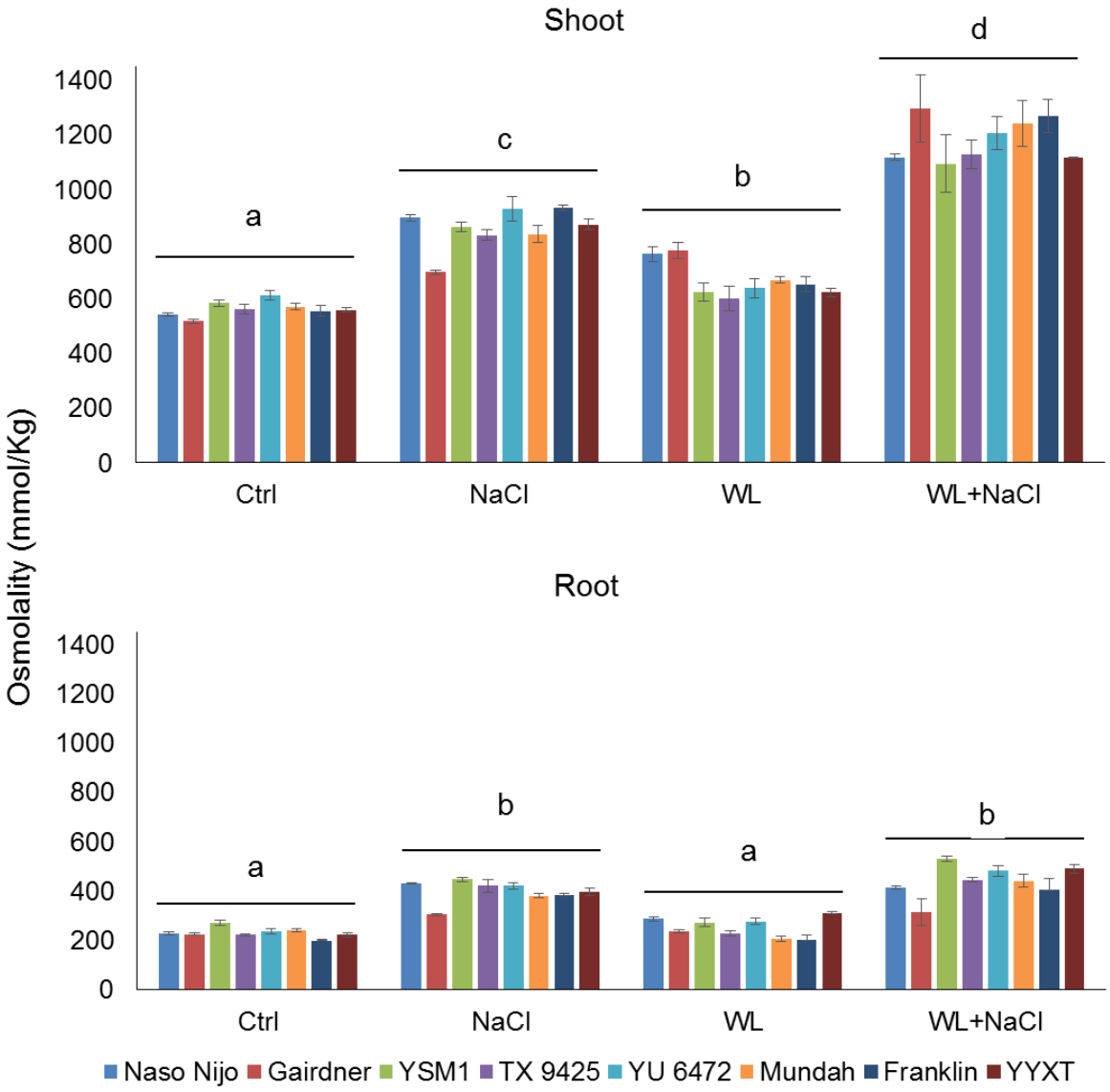

Figure 5.11. Effects of separate and combined stresses of salinity and waterlogging on shoot and root osmolality of selected 8 barley varieties under hydroponic conditions. 6 day old seedlings were subjected to one of the four treatments; Control (No NaCl, well drained), NaCl (irrigated by 150mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 150mM NaCl solution). Plants were harvested after 8 days stress for osmolality measurements. The lower case letters indicate the significant difference between treatments (averaged for all 8 varieties at $P<0.01$), the error bars indicate the standard error of all replicated for each treatment/variety

Shoot osmolality was higher than root osmolality in all control and treated plants in general. Shoot osmolality was 2-3 fold higher than root osmolality in the control plants. YSM1 and Franklin had the least and most ratio of shoot to root osmolality difference, with the average of 220% and 278% respectively. Whereas shoot osmolality of NaCl treated plants

was 1.3 to 1.8 fold higher than control, they had similar ratios of shoot to root osmolality. The shoot osmolality of NaCl treated varieties were 1.8 to 2.5 fold higher than root osmolality. YSM1 and Franklin had the least and most difference with the average percentage of 193% and 244% respectively. Barley varieties under WL stress after 8 days had a greater difference between shoot and root osmolality, which ranged from 2 to 3.2 fold among varieties. YYXT and Franklin had the least and most difference respectively. WL/NaCl had the most effect on the shoot and root osmolality ratios with 1.6 to 6.2 fold more shoot osmolality than root osmolality. YSM1 and Gairdner had the least and most difference respectively with the average of 188% and 528% respectively. Shoot osmolality was more affected by stresses than root (Figure 5.11).

Table 5.11. The average shoot and root osmolality of selected 8 barley varieties relative to control (%) after 8 days of separate and combined NaCl and WL stresses

Cultivar/ Treatment	Osmolality (% Control), Shoot			Osmolality (% Control), Root		
	NaCl	WL	WL/NaCl	NaCl	WL	WL/NaCl
Naso Nijo	165	136	199	190	127	182
Gairdner	135	150	250	135	105	140
YSM1	148	107	187	165	101	196
TX9425	148	107	201	189	103	200
YU6472	152	105	198	179	117	204
Mundah	147	117	217	157	85	182
Franklin	168	117	228	192	102	203
YYXT	156	111	199	178	140	220

Table 5.12. Range of sap osmolality under single and combined stresses of 150mM NaCl and waterlogging (mOsmol kg–1)

	Control	**NaCl**	**WL**	**WL/NaCl**
Shoot	519-611	698-982	595-777	1037-1297
Root	200-271	305-448	204-335	316-522

The table below illustrates the correlation between shoot and root osmolality of 8 varieties of barley from a range of sensitive to tolerant to salinity under separate and combined NaCl and WL stresses besides the control plants. It is apparent from the table that root osmolality of control and stressed plants all had a positive correlation. Plant shoots under NaCl treatment had a positive correlation with control shoot and negative correlation with WL and WL/NaCl stresses. WL and WL/NaCl stressed plants had a positive correlation for shoot osmolality and they both had negative correlation with control and salinity treated shoot osmolality (Table 5.13).

Table 5.13. Correlation between the effects of NaCl, WL and WL/NaCl stresses on plant shoot and root osmolality after 8 days

		Shoot				**Root**		
		Control	**NaCl**	**WL**	**WL/NaCl**	**Control**	**NaCl**	**WL**
Shoot	**NaCl**	0.688**						
	WL	-0.288	-0.551**					
	WL/NaCl	-0.317	-0.148	0.232				
Root	**Control**	.490**	-0.094	-0.180	-0.265			
	NaCl	.592**	0.656**	-0.479**	-0.616**	0.479**		
	WL	0.143	0.103	-0.049	-0.320	0.175	0.308	
	WL/NaCl	0.443*	0.413*	-0.458*	-0.649**	0.329	0.591**	0.338

The results of correlational analysis of shoot to root osmolality for control plants, separate and combined NaCl and WL stresses are set out in Figure 5.12. There is a significant

(P<0.01) positive correlation between shoot and root osmolality of control plants. Plants under NaCl stress after 8 days of treatment showed significant positive correlation (P<0.01) between root and shoot. Plants under 8 days WL stress had no significant correlation between shoot and root. The correlation between shoot and root osmolality under WL/NaCl stress was a significant (P=0.01) negative correlation. It is interesting that there was the same strength of correlation but a reversal of the relationship between root and shoot osmolality of plants treated with WL/NaCl compared to NaCl. (Figure 5.12).

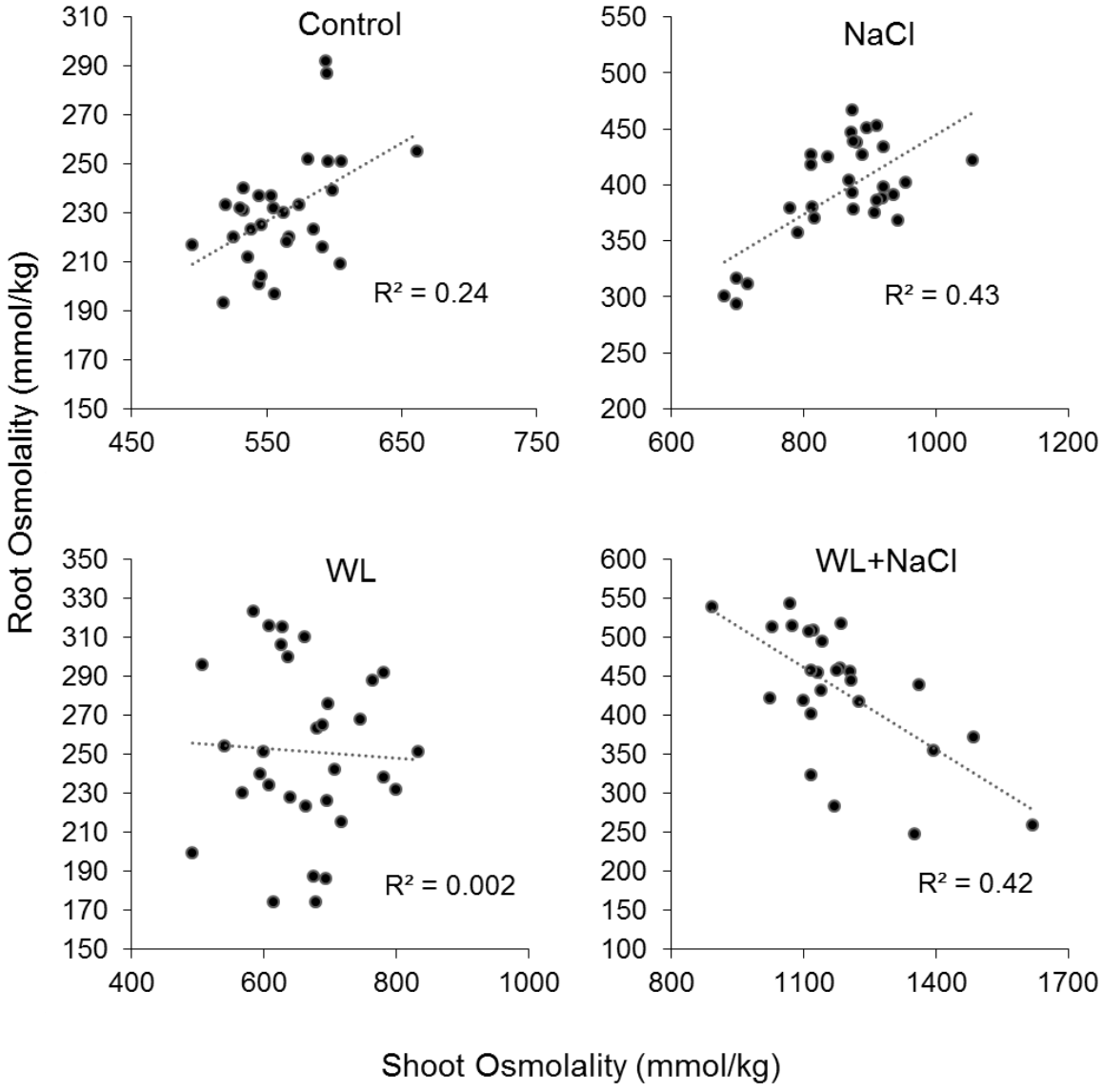

Figure 5.12. The correlation between shoot and root osmolality after 8 days of separate and combined NaCl and WL stresses

The Pearson correlation analysis was used to determine the relationship between shoot and root osmolality of control and all stressed plants. There was no significant correlation between absolute numbers of shoot osmolality of the plants under WL/NaCl stress compared to separate stresses and control plants. While there was a good significant ($p<0.01$) correlation between shoot osmolality of WL and WL/NaCl treated plants relative to the control (Figure 5.13). As displayed above in Table 5.13, there was a good significant correlation between root osmolality of plants under combined WL/NaCl stress and NaCl. The relative osmolality of roots for the same pair of stresses were also significantly ($p<0.01$) correlated.

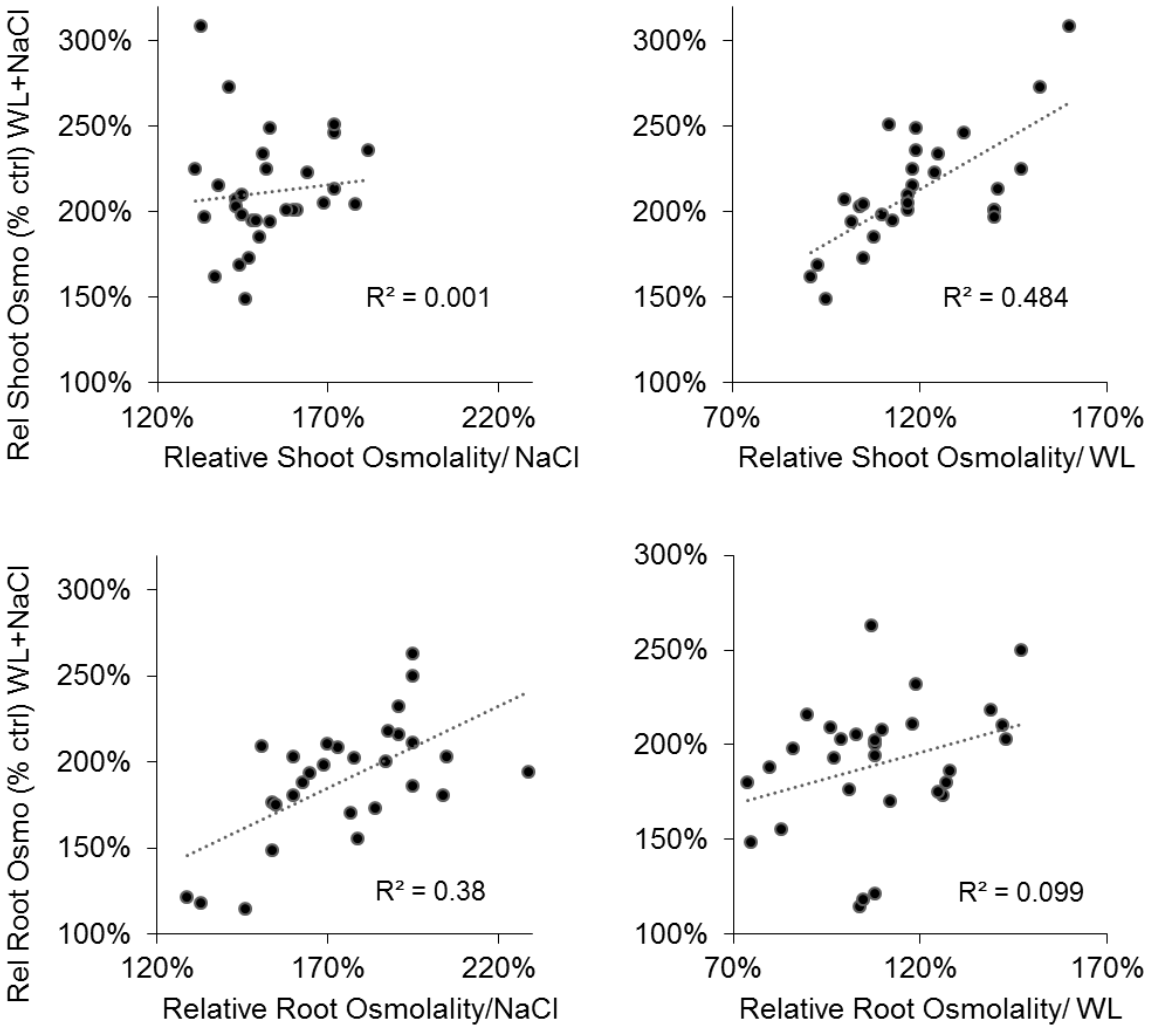

Figure 5.13. The correlation between relative shoot and root osmolality to their control under separate and combined NaCl and WL stresses.

The correlation between shoot osmolality and relative water content of the plants at the same time of osmolality sampling and 8 days after is shown in Table 5.14. The relative water content of plants under 16 days of stress showed a stronger correlation with osmolality than plants under 8 days stress although osmolality measurements are from 8 days stressed plants only. It can be concluded that osmolality can predict the effect of stress on the relative water content. The results obtained from correlation analysis presented that NaCl and WL/NaCl have negative correlation between osmolality and relative water content but WL stressed plants have a positive correlation (Table 5.14).

Table 5.14. Correlation between the shoot osmolality and relative water content (RWC) under NaCl, WL and WL/NaCl stresses

		Osmolality			
		Control	**NaCl**	**WL**	**WL/NaCl**
1st RWC	**Control**	-0.288			
	NaCl		0.014		
	WL			-0.025	
	WL/NaCl				0.016
2nd RWC	**Control**	-0.637**			
	NaCl		-0.397*		
	WL			0.490**	
	WLNaCl				-0.487*

5.2.5 Na^+ and K^+ Content

5.2.5.1 Na^+ Content

WL/NaCl stress increased shoot and root Na^+ content of the four selected barley varieties regardless to their tolerance to salinity although root Na^+ content under NaCl was higher than WL/NaCl. There was no significant difference between Na^+ content of the root under control and WL stress. Na^+ content of the shoot was significantly different between all four treatments. Control plants had the lowest Na^+ content and plants under WL/NaCl stress had the highest (Figure 5.14).

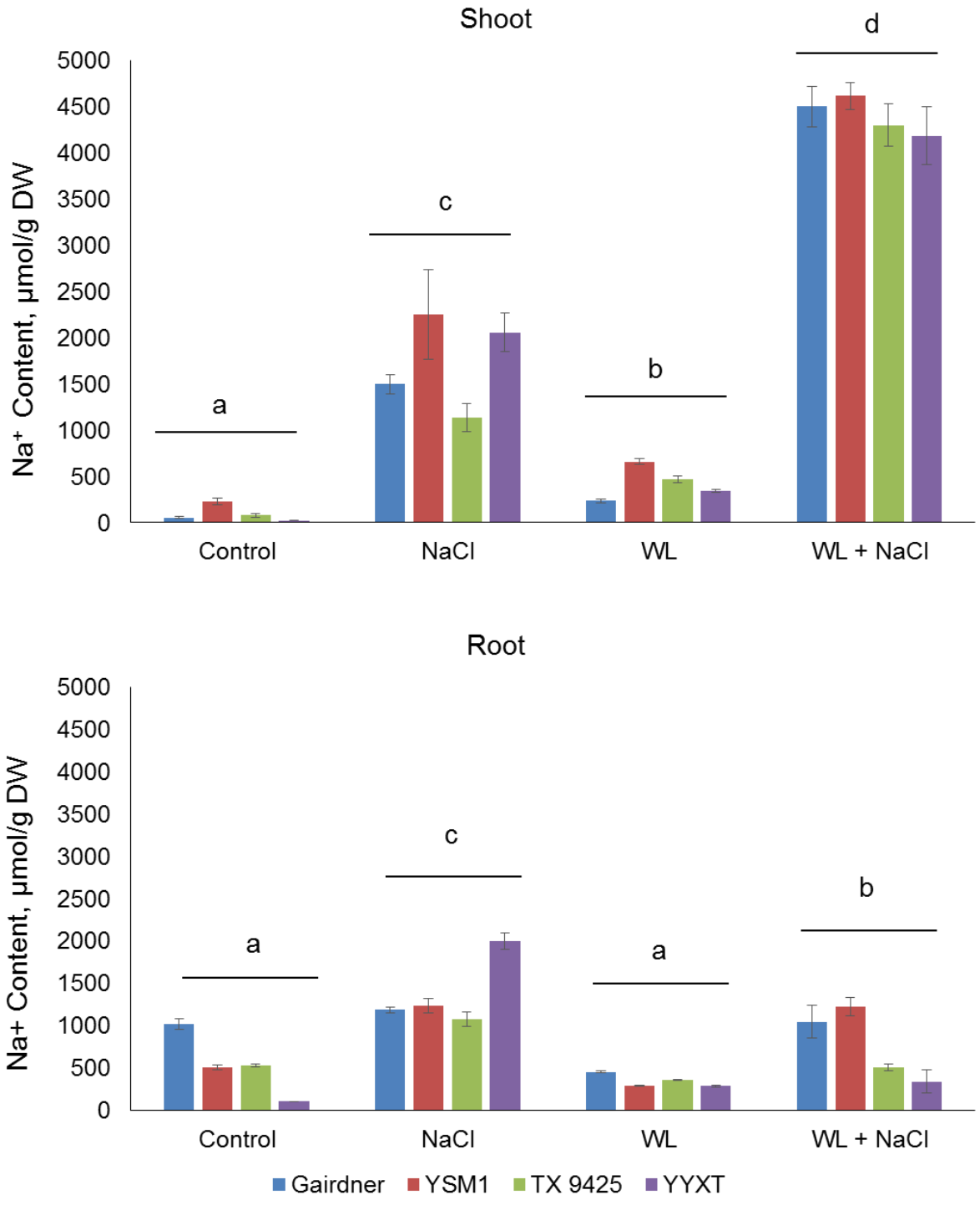

Figure 5.14. Effects of separate and combined stresses of salinity and waterlogging on shoot and root Na^+ content of selected 8 barley varieties under hydroponic conditions. 6 day old seedlings were subjected to one of the four treatments; Control (No NaCL, well drained), NaCl (irrigated by 150mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 150mM NaCL solution). Plants were harvested after 16 days stress for Na^+ content measurements. The lower case letters indicate the significant difference between treatments (averaged for all 8 varieties at $P<0.01$), the error bars indicate the standard error of all replicated for each treatment/variety

The average shoot Na^+ content for the selected varieties after 16 days of stress was 998% to 9003% under NaCl stress, 292% to 1497% under WL stress and 2044% to 18304% under WL/NaCl stress relative to the control. YSM1 and YYXT had the least and most increase for all three stresses, respectively. The average root Na^+ content was 116% to 1924% (variety low variety high) under NaCl stress, 45% to 277% (Gairdner and YYXT) under WL and 97% to 330% (TX9425 and YYXT) under WL/NaCl relative to the control (Table 5.15).

Table 5.15. The average shoot and root Na+ content of selected 4 barley varieties relative to control (%) after 16 days of separate and combined NaCl and WL stresses

Cultivar/ Treatment	Na^+ Content (% Control), Shoot			Na^+ Content (% Control), Root		
	NaCl	WL	WL/NaCl	NaCl	WL	WL/NaCl
Gairdner	2456%	432%	6639%	116%	45%	103%
YSM1	998%	292%	2044%	243%	57%	241%
TX9425	2254%	592%	5473%	203%	68%	97%
YYXT	12627%	1754%	21450%	1924%	277%	330%

5.2.5.2 K^+ Content

All stresses significantly reduced shoot and root K+ content. The highest shoot K^+ content reduction was for the plants under WL/NaCl and NaCl stress, the reduction under WL was intermediate. The highest root K^+ content reduction was for the plants under WL/NaCl stress followed by NaCl and then WL (Figure 5.15). WL/NaCl stress decreased shoot and root K^+ content of the four selected barley varieties regardless to their tolerance to salinity.

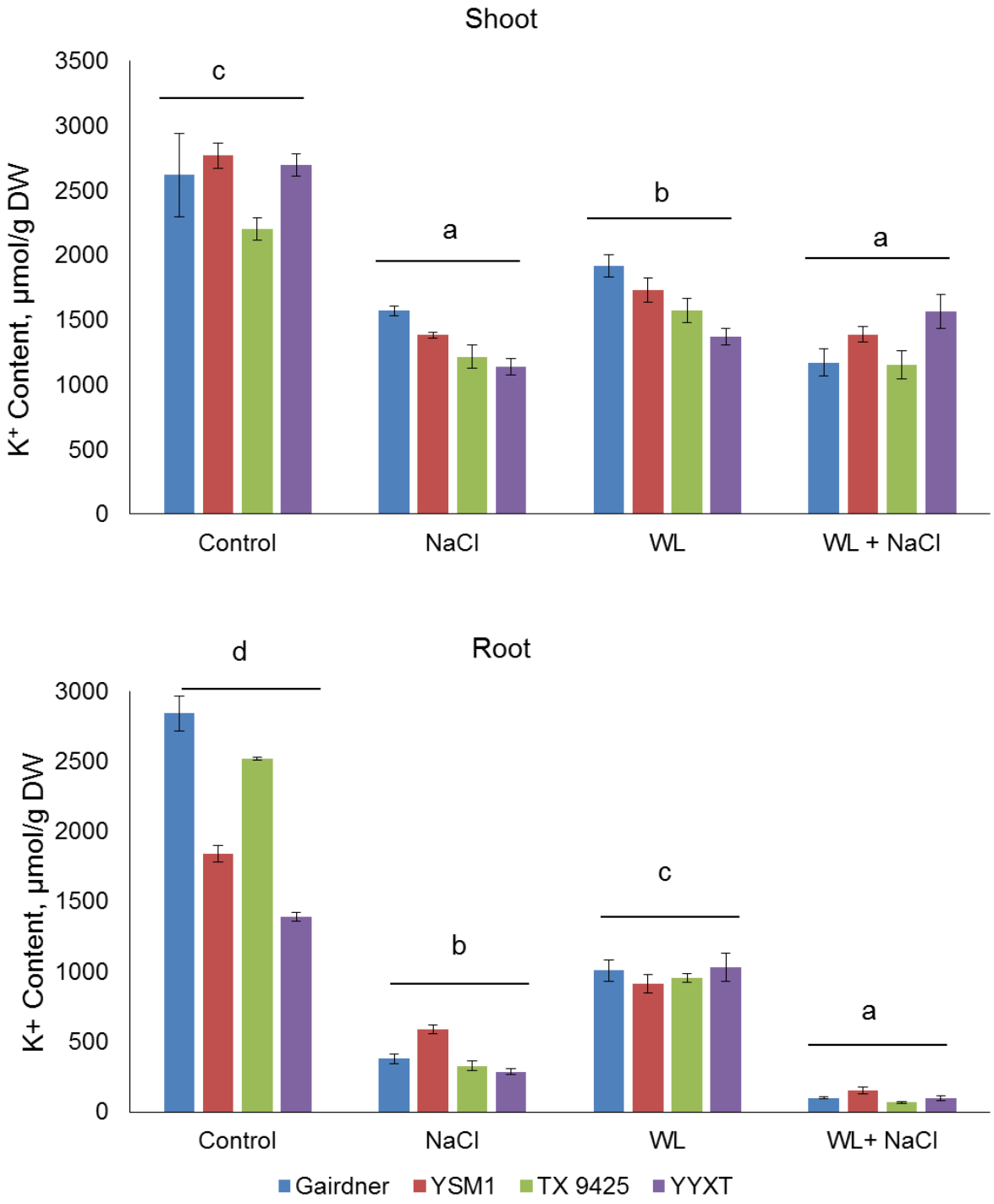

Figure 5.15. Effects of separate and combined stresses of salinity and waterlogging on shoot and root K^+ content of selected 8 barley varieties under hydroponic conditions. 6 day old seedlings were subjected to one of the four treatments; Control (No NaCL, well drained), NaCl (irrigated by 150mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm), NaCl/WL (submerged by 150mM NaCL solution). Plants were harvested after 16 days stress for K^+ content measurements. The lower case letters indicate the significant difference between treatments (averaged for all 8 varieties at $P<0.01$), the error bars indicate the standard error of all replicated for each treatment/variety

The average shoot K^+ content under NaCl was 42% to 60% and under WL was 51% to 73% relative control, YYXT and Gairdner had the least and most increase, respectively. The average shoot K^+ content under WL/NaCl was 45% to 58% relative to control, Gairdner and YYXT had the least and most increase respectively. The average root K^+ content under NaCl was 13% to 32% relative to control, Gairdner and TX9425 had the least and YSM1 had the most increase. The average root K^+ content under WL was 35% to 74% relative to control, Gairdner and YYXT had the least and most increase, respectively. The average root K^+ content under WL/NaCl was 3% to 8% relative to control, Gairdner and YSM1 had the least and most increase, respectively.

Table 5.16. The average shoot and root K+ content of selected 4 barley varieties relative to control (%) after 16 days of separate and combined NaCl and WL stresses

Cultivar/ Treatment	K^+ Content (% Control), Shoot			K^+ Content (% Control), Root		
	NaCl	WL	WL/NaCl	NaCl	WL	WL/NaCl
Gairdner	60%	73%	45%	13%	35%	3%
YSM1	50%	63%	50%	32%	50%	8%
TX9425	43%	55%	41%	13%	38%	3%
YYXT	42%	51%	58%	21%	74%	7%

5.3 Discussion

According to the classical view (Munns 2002; Munns, Schachtman et al 1995) the osmotic component of salt stress dominates over the first 2-3 weeks, after which specific effects of Na^+ toxicity in the shoot become the major constrain. While this view is somewhat an oversimplification and does not account for the severity of the stress per se, it may help explain why genotypes contrasting in their salinity tolerance did not show major differences in growth rate under short-term (16 days) salinity exposure. These cultivars were previously classified as salt-sensitive and salt-tolerant based on the damage index under extreme (300 mM naCl) conditions applied for one month (Zhu et al 2015), e.g. based on their tissue tolerance (but not osmolality-tolerance). As a result, the fast growing barley variety Naso Nijo grew fast but showed reduced chlorophyll content. At the same time, a tolerant variety TX9425 grew slower but was less affected by salt effects (Figure 5.1, Figure 5.3 and Figure 5.9). It is interesting that TX9425 had the most reduction in FW after 8 days under waterlogging stress but it had a greater recovery over the next 8 days to be in the upper half of varieties in terms of FW after 16 days of treatment. TX9425 had the same trend under combined WL/NaCl stresses. These findings are also consistent with the classical yield/tolerance trade-off. As the aim of this experiment was not to study the effects of salinity but the combination of salinity with waterlogging, 16 days stress was enough to show the barley varieties tolerance to combined WL/NaCl stress.

It has been shown that WL impacts on traits such as shoot FW and shoot osmolality are more correlated to WL/NaCl compared to NaCl (Figure 5.2 and Figure 5.12) and possible reasons for these observations are discussed below.

Control of xylem ion loading and Na^+ delivery to the shoot is an effective factor in tolerance to combined WL/NaCl stress

Salinity stress conditions around the root results in root tissue depolarization (Wegner et al. 2011) along with continuous Na^+ accumulation in parenchyma cells. Parenchyma cells cytosolic Na^+ concentration will increase due to ongoing depolarization (because of continuous Na^+ entering) while xylem Na^+ concentrations are still low. Channel-mediated xylem Na^+ loading becomes possible because of these two factors. Then, Na^+ is sent to the shoot by the transpiration stream for the rapid osmotic adjustment. As xylem Na^+ concentration increases, the parenchyma cells become repolarized therefore passive loading

could not be continued. When the passive loading is stopped, plants have to switch to possible thermodynamically active xylem Na^+ loading, SOS1 Na^+/H^+ exchanger and Cation-Cl (CCC) co-transporter. SOS1 is specially expressed at the xylem symplast boundary of roots in glycophytes (Shi et al. 2002). This exchanger was reported to be active under salinity stress in both glycophytes (Shi et al. 2002) and halophytes (Cosentino et al. 2010; Oh et al. 2010). CCC proteins are secondary active transporters that arbitrate the transport of Cl- and K^+ and/or Na^+ through the plasmalemma (Haas 1989). The negative membrane potential results in thermodynamically passive Cl- movement from xylem parenchyma to the xylem and it will be a driving force to load Na^+ alongside against the electrochemical potential. This loading through CCC transporters makes K^+ loading into the xylem feasible with Na^+ (Shabala et al. 2010) and helps to maintain the xylem Na^+/K^+ ratio that provides the optimum osmotic adjustment in the shoot.

K^+ retention in roots is most essential for combined stress tolerance

Waterlogging and salinity stresses affect plants via a complex network of signal transduction pathways. Salinity *per se* disturbs plants performance by salt-specific and osmotic stresses (Hasegawa et al. 2000). Waterlogging signalling happens through a wide range of secondary messengers and also oxygen- and ethylene-dependent pathways (Visser and Voesenek 2005). The proposed common dominator for both of these two stresses is: stress-induced membrane depolarisation (Barrett-Lennard and Shabala 2013). NaCl-induced depolarisation for a number of plant species has been stated from 40 to 80mV (Smethurst et al. 2008; Cuin et al. 2008; Shabala et al. 2007; Chen et al. 2005; Laurie et al. 2002; Horie et al. 2001; Cakirlar and Bowling 1981). Besides, a comparable magnitude depolarisation is expected from oxygen deprivation by itself (Buwalda et al. 1988b; Cheeseman and Hanson 1979; Contardi and Davis 1978). While there is not enough information about the magnitude of plasma membrane depolarisation for combined waterlogging and salinity stress, it is assumed that oxygen deficiency will compromise the capacity of plants to rise the plasma membrane H^+ ATPase activity to restore (otherwise depolarise) membrane potential under saline conditions (Barrett-Lennard and Shabala 2013). Furthermore, the reported salinity-induced activation capacity of H^+-ATPase activity (Hasegawa et al. 2000) might be lost under waterlogging conditions with severe consequences to intracellular K^+ homeostasis.

Reduced K^+ content in plants under combined WL/NaCl stresses (Figure 5.15), noting the key role of H^+-ATPase activity and voltage gating, could be explained by either increased

K^+ efflux via depolarisation-activated outward-rectifying (KOR) K^+ channels, or by decreased root K^+ uptake via inward-rectifying (KIR) K^+ channels (Shabala 2003; Véry and Sentenac 2002). Based on literature, it is proved that activation of KOR channels is responsible for salinity-induced K^+ deficiency in plant tissue under saline conditions (reviewed by (Shabala and Cuin 2008)). In regards to the strong non-linearity of KOR channel characteristics (Shabala et al. 2006), it is assumed that even minor additional depolarisation triggered by hypoxia results in an intense increase in net K^+ efflux under the combined stresses (Barrett-Lennard and Shabala 2013)

Shoot Na^+ content determines plant tolerance to combined stress

The effects of combined WL/NaCl stress on shoot Na^+ content is much more severe than on the root. Shoot Na^+ content of the plants under WL/NaCl stress was higher than under NaCl stress while root Na^+ content of plants under WL/NaCl was less than under NaCl stress alone (Figure 5.14). A plants ability to restrict Na^+ in the shoot determines their tolerance to combined WL/NaCl stress. As explained above, controlling Na^+ xylem loading from the shoot is one mechanism but also increased Na^+ uptake by roots or a reduced root ability to extrude Na^+ back into the external media will affect shoot Na+

Under combined WL/NaCl stress, shoot Na^+ and Cl^- concentrations were minimized by lower xylem concentrations entering the shoot leading to higher shoot dry mass in *L. tenuis* (tolerant to WL/NaCl) compared with *L. corniculatus* (sensitive to WL/NaCl) (Teakle et al. 2010). NHX, the Na^+/H^+ antiporter that is the candidate gene for maintenance of low shoot Na^+, is affected by waterlogging conditions because this ion transporters activity is reliant on O_2 for ATP activity to maintain H^+ gradient across membranes.

Nevertheless, it is assumed that the electrochemical gradient for Na^+ entry through NSCC is reduced by root plasma membrane depolarisation under waterlogging conditions (NSCC as a key pathway for Na^+ uptake; (Demidchik and Maathuis 2007)). Consequently, a decrease in the root's ability to extrude Na^+ back into external media is most likely the reason (Barrett-Lennard and Shabala 2013). Plasma membrane Na^+/H^+ antiporters that are fuelled by plasma membrane H^+-ATPase are responsible for active Na^+ transport outward (Shi et al. 2000; Qiu et al. 2003). These exchangers activity is recognized to be pH-balanced (Oh et al. 2010) while cytosol hypoxia-induced acidification is broadly reported (Felle 2005).

Chapter 6: Net K^+ and H^+ fluxes from barley roots exposed to salinity and hypoxia stress and their combination

6.1 Introduction

Progress in plant breeding to combine WL/NaCl stress is currently handicapped by two major factors. The first is the understanding of molecular mechanisms and downstream targets mediating plant adaptive responses, and the second is the lack of convenient mechanism-based screening methods for this trait. Until now, such screening was conducted mainly in the field (which is time-consuming and environment-dependent). The main measured traits used were growth rate, plant survival, germination rate, root and shoot elongation rate, chlorophyll content, damage index based on chlorosis and necrosis, shoot and root Na^+, K^+ content and K^+/Na^+ ratio. Neither of them is directly related to a function of one specific gene but most likely reflect the overall feedback responses of the organism to the stressor.

In addition to controlling Na^+ uptake, maintaining cytosol K^+ content is another key feature in plant tolerance to WL/NaCl. Nonetheless, it is important to not confuse cytosolic and whole plant K^+ and Na^+ content. The measured Na^+ and K^+ content from plant sap or dried material represents a mix of cytosolic and vacuolar compartments and, hence, does not account for differential genotypic ability to sequester Na^+ in the vacuole. Neither of them accounts for tissue-specific operation of the candidate genes.

The above limitation can be largely overcome if net fluxes of ions are measured from appropriate root zones. In this context, the MIFE technique for non-invasive ion flux measurements represents an excellent tool to study specificity of root responses to salinity, hypoxia, and their combination. Given our previous findings (see Chapter 5) that root K^+ content was a critical factor behind contrasting tolerance to combined stress, in this chapter we have focused on measuring K^+ fluxes from stress-exposed barley roots. We also measured H^+ flux as a proxy for H^+-ATPase activity.

6.2 Results

Five day old seedlings which were stressed for 2 days were used in MIFE experiments. Plants were already in the measuring chambers for two days under hypoxia and hypoxia/NaCl stress, therefore they were just transferred to the green light area for 30 min adaptation before the experiments. Control and NaCl stressed seminal intact root from the whole seedling was immobilized in a measuring chamber with the help of silicon stoppers and the chamber was filled with BSM for control and BSM+150mM NaCl for NaCl stressed plants (for more details please see chapters 3.3.5 and 3.3.6)

NaCl stress resulted in higher K^+ efflux from the mature root zone of ZUG403 (-32 $nmolm^{-2}s^{-1}$) compared to Gebeina (-6 $nmolm^{-2}s^{-1}$) while YU6472 showed a minor uptake but no efflux (0.1 $nmolm^{-2}s^{-1}$) under 150mM NaCl stress. When measurements were performed on intact plants, with coleoptiles protruding in the air, hypoxia stress had a robust effect on K^+ efflux of ZUG403, reducing it to -190 $nmolm^{-2}s^{-1}$ while in Gebeina K^+ flux was -32 $nmolm^{-2}s^{-1}$ and YU6472 (most tolerant variety) had a low K^+ uptake of about 9 $nmolm^{-2}s^{-1}$. When plant coleoptiles were excised, combined stress resulted in a massive K^+ efflux from the mature root zone of ZUG403 (-182 $nmolm^{-2}s^{-1}$) while Gebeina and YU6472 both showed minor uptake, 0.1 and 4 $nmolm^{-2}s^{-1}$, respectively.

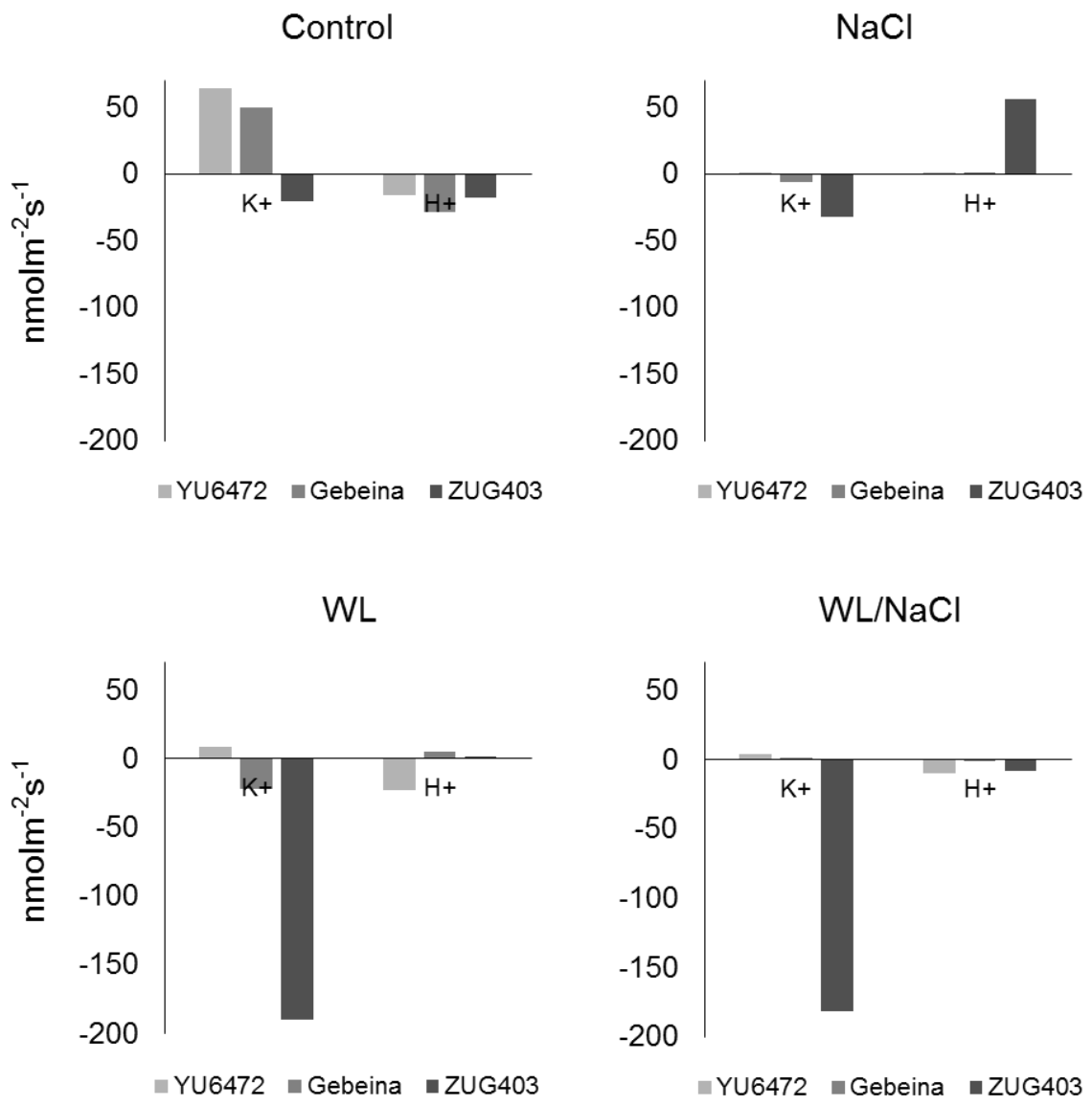

Figure 6.1. Effects of separate and combined stresses of salinity and waterlogging on K^+ and H^+ flux measurements from the mature root zone (1 cm from the coleoptile as shown in Figure 3.13) of five day old barley seedling under hydroponic conditions. Three day old seedlings were subjected to one of the four treatments for two days; Control (No NaCl, well drained), NaCl (irrigated by 150mM NaCl solution, well drained), Waterlogging (submerged under tap water by 1 cm) and NaCl/WL (submerged by 150mM NaCl solution), the data are the mean of 6 replicates

Oxygen availability is an important element of root growth and functioning under hypoxic conditions and restricted oxygen supply results in an immediate K^+ loss from roots. It was demonstrated that roots of intact barley were able to maintain H^+-pumping activity under hypoxic conditions when their coleoptiles were protruding in the air (Zeng et al 2014) while after cutting the coleoptile, and disrupting oxygen transport from the shoot to root, a

stronger membrane depolarization was observed due to oxygen-limited conditions. Accordingly, we have compared responses from intact seedlings and seedlings with excised coleoptiles, to salinity and hypoxia stress and their combination (Figure 6.2). Intact barley seedlings were subjected to hypoxia and combined hypoxia/NaCl stresses for 48 hours, and steady-state H^+ and K^+ fluxes were measured. Then their coleoptile was cut to assess if the oxygen taken through the coleoptile was affecting the plants ion flux values. As it is shown in Figure 6.2 all three barley intact varieties showed K^+ uptake under hypoxia stress and they started leaking K^+ after cutting off the coleoptile. The K^+ leakage of ZUG403, Gebeina and YU6472 under hypoxia stress increased by 2, 11 and 16 fold after cutting the coleoptile, respectively. Even though ZUG403 K^+ efflux was 2 and 3 fold more than YU6472 and Gebeina after cutting the coleoptile. The K^+ leakage of ZUG403 under hypoxia/NaCl increased by 13 fold after cutting the coleoptile while it remained relatively unchanged in Gebeina and YU6472.

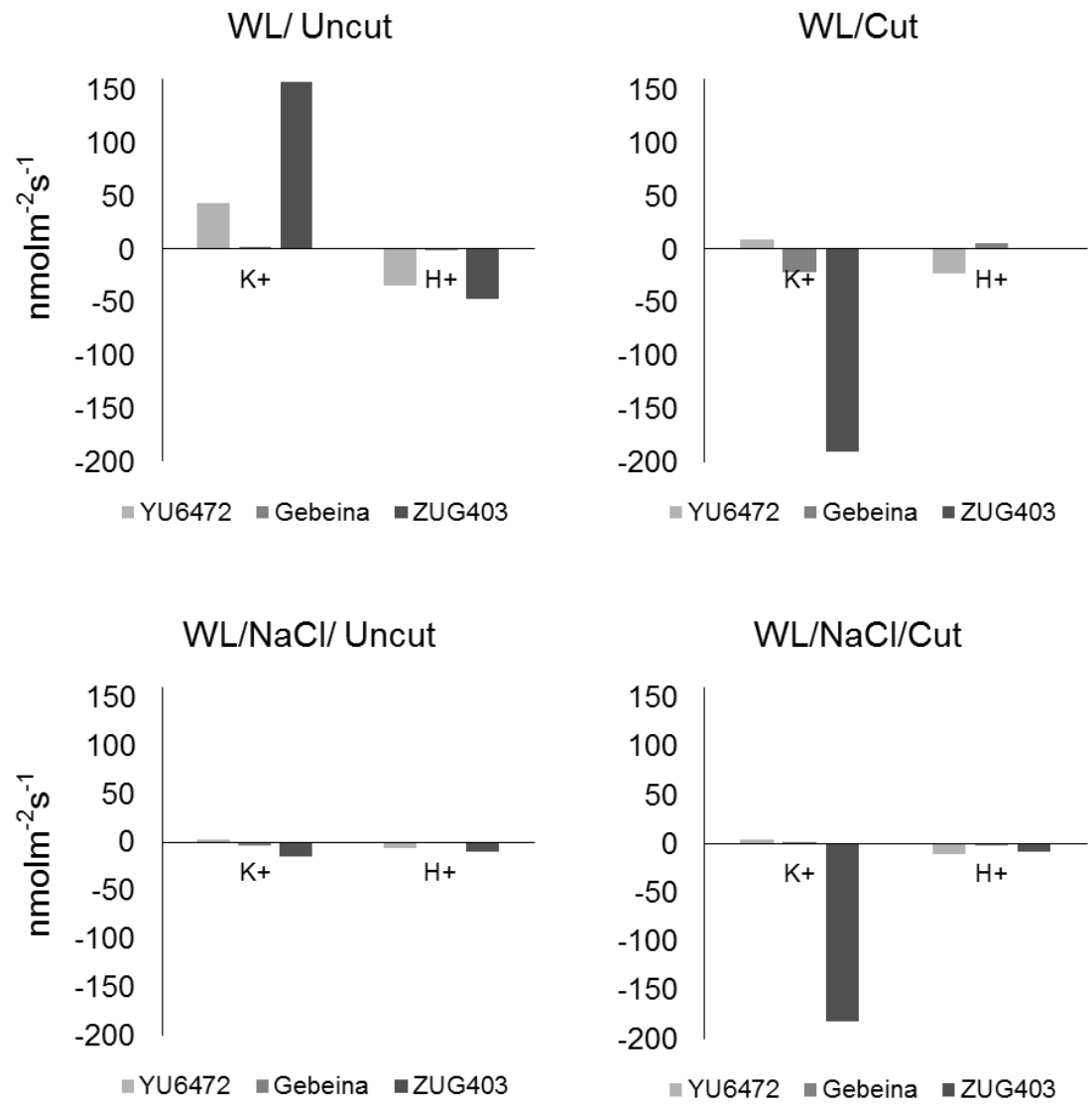

Figure 6.2. K^+ and H^+ flux measurements of barley plants mature root zone under hypoxia and combined hypoxia/150 mM NaCl stresses. Uncut – intact plants with coleoptile protruding into the air; Cut - plants with coleoptiles excised, the data are the mean of 6 replicates

6.3 Discussion

Root K^+ efflux is affected by hypoxia

It was shown in section 4 that tolerance to WL/NaCl stress is determined mostly by sensitivity to WL. In section 5 it was shown that K^+ retention in roots was the most essential for combined stress tolerance. The current study on mature root zone K^+ flux measurements by MIFE has confirmed that K^+ efflux is more strongly decreased by hypoxia compared to NaCl and the K^+ reduction rate under hypoxia/NaCl stress is in the same range as hypoxia. YU6472, as a more tolerant variety to hypoxia/NaCl, did not show K^+ efflux compared to ZUG403, a sensitive variety to hypoxia/NaCl, that had -182 $nmolm^{-2}s^{-1}$ K^+ efflux (Figure 6.1).

Plants need to retain K^+ in the cytosol while limiting Na^+ influx to tolerate WL/NaCl stress (Barrett-Lennard and Shabala 2013). Voltage dependend channels such as GORK consititutes the major pathways for K^+ efflux (Ache et al. 2000). These channels are activated by depolarization due to Na^+ influx into the cytosol under WL/NaCl stress. H^+ efflux can balance the voltage difference across the membrane to reduce K^+ efflux. At the plasma membrane, this H^+ efflux is provided H^+-ATPase pumps, even though their activity is greatly limited under waterlogging conditions due to the onset of anerobic respiration (see chapter 4.3 for more details). In the current experiment, the measured H^+ flux could be used as a proxy for the H^+-ATPase pump activity that is responsible for membrane potential maintanance. Therefore, due to voltage dependant activity of K^+ efflux channels like GORK, and their activation by depolarization, reduced H^+ pumping should be correlated with root K^+ loss. As it is seen in Figure 6.1, under hypoxia stress, the tolerant variety YU6472 showed the highest H^+ efflux (-23 $nmolm^{-2}s^{-1}$) and as a result it was able to maintain K^+ in the cytosol and had a low rate of K^+ influx (9 $nmolm^{-2}s^{-1}$) but not any efflux. This is in comparison to ZUG403 that had no H^+ efflux (1 $nmolm^{-2}s^{-1}$) and as a cosequence had a large K^+ efflux (-190 $nmolm^{-2}s^{-1}$).

Oxygen is transferred to the root from coleoptile to reduce the adverse effects of hypoxia

The high ethylene concentrations of the plants under waterlogging *per se* stress induced acclimation to waterlogging by development of aerenchyma and the elongation of coleoptiles called ‘snorkles’ (Jackson 1985; Kordan 1974). The importance of aerenchyma formation in

plants exposed to waterlogging and WL/NaCl conditions regarding gas exchange between aerial and flooded organs has already been dicussed in section 4.3. Though there is not much information about the elongation of coleoptiles under WL/NaCl stress. The MIFE experiments reported here have shown that plants may also achieve the same goal to fule H^+ pumps in root tissues and reduce adverese effects of hypoxia and its combination with salinity by increased oxygen supply from the coleptile (shoot).

Chapter 7: General Discussion

This project has significantly increased the understanding of the physiology of the interaction between salinity and waterlogging on barley plants through detailed studies with three sets of experiments including plant growth under glasshouse soil and hydroponic conditions followed by ion flux measurements by MIFE.

The limited energy production due to waterlogging stress under WL/NaCl conditions has to be divided in adaptive responses to both waterlogging and salinity stress. In order to overcome oxygen deficiency, plants are reported to enact several anatomical and morphological mechanisms such as aerenchyma formation and formation of adventitious roots. The current study has shown that aerial parts of the plant are a pathway for delivering oxygen under saline hypoxic conditions.

Plants are required to manage the osmotic adjustment and then maintain cellular K^+/Na^+ homeostasis to tolerate salinity under hypoxic conditions. As Na^+ content in apoplast is much higher than cytoplasm in the saline hypoxic conditions, Na^+ is entered to the cytoplasm by passive influx through NSCC. In order to balance passive Na^+ influx, plants have to maintain the Na^+ efflux either across the plasma membrane back into apoplast or across the tonoplast into the vacuole (Amtmann and Beilby 2010).

NHX antiporters in the tonoplast are able to influx Na^+ into the vacuole. The required H^+ for these Na^+/H^+ exchangers can be provided either by ATPase or PPase (Fukuda et al. 1998). As ATPase activity is limited under hypoxic conditions, it is suggested that PPase are more functional under combined WL/NaCl in tonoplast.

In the plasma membrane, Na^+/H^+ SOS1 antiporters are named to be responsible for Na^+ efflux to the apoplast. The required driving force to operate these exchangers is provided by the H^+-ATPase; however, its activity is limited under hypoxic conditions. The possible alternative "fuel source" may be H^+-PPase. Although H^+-PPase pumps so far have meen reported to reside mainly in tonoplast, it cannot be excluded that some of them may also operate at the plasma membrane (at least, temporarily). Is this is the case then plant breeding to WL and combined WL/NaCl stress tolerance should be focused of increasing the number of functional H^+-PPases at the plasma membrane.

Plants are required to maintain a positive balance between promoting Na^+ efflux and limiting K^+ efflux in cytoplasm to tolerate combined stresses. There are two main possible pathways for K^+ efflux out of the root cells including NSCC and GORK that are activated under combined WL/NaCl conditions:

NSCC, the non-selective channels, will influx Na^+ as well as efflux K^+ under saline hypoxia conditions. These channels are activated by ROS produced under hypoxic conditions from mitochondria. Plants can implement mechanisms such as scavenging ROS to avoid ROS activation of the channels (Figure 7.1).

GORK channels are activated by membrane depolarization and respond to the voltage difference across the membrane. Therefore, Na^+ entering the cytoplasm from NSCC channels will activate GORK channels by depolarizing the membrane. ROS production by mitochondria is another activation trigger for GORK channels. H^+ efflux out of the cytoplasm can deactivate K^+ efflux. The required H^+ can be provided either from ATPase and PPase as explained earlier (Figure 7.1).

It is shown in the current study that K^+ efflux in the root is the key factor in barley tolerance to WL/NaCl. The presence of a pump, such as PPase in the plasma membrane, that is able to provide the required H^+ for both Na^+ efflux through SOS1 but also limiting GORK activity is suggested (Figue 7.1).

By examining the effects of combined WL/NaCl on the whole plant in soil and under hydroponic conditions followed by ion exchange studies on the roots by MIFE, it has been demonstrated that:

- The effects of combined WL and NaCl stress is more severe than either NaCl or WL stress alone
- The combined effects are synergistic but not additive
- Tolerance to WL/NaCl stress is determined mostly by tolerance to WL
- K^+ ionic relations is more critical in explaining the tolerance to WL/NaCl stress compared Na^+ ion relations
- Control of xylem ion loading and Na^+ delivery to the shoot is an effective factor in tolerance to combined WL/NaCl stress
- K^+ relation in roots is the most critical factor in tolerance to WL/NaCl stress
- Shoot Na^+ content determines plant tolerance to WL/NaCl stress

- Root K^+ efflux is more affected by waterlogging
- Oxygen is transferred to the root from coleoptile to reduce the adverse effects of hypoxia

The future directions from this work should focus on exploring the possibility of creating a functional H^+- PPases at the root plasma membrane. Another avenue would be to investigate aspects of tissue-specific regulation of ion transport activity in stressed roots, linking it with oxygen availability. To make the results applicable to field studies the effect of other constrains observed in waterlogged soils (such as elemental toxicity and toxic effects of various secondary metabolites) should be studied for their possible synergistic interaction with salinity. In this context, of a special interest is Fe^{3+} - a transition metal that is accumulated in flooded soils (Shabala et al 2014) and that could interact with hydrogen peroxide in the root apoplast to produce highly aggressive hydroxyl radical (Demidchik et al 2014; Rodrigo-Moreno et al 2013). When applied to roots, hydroxyl radicals induce massive K^+ efflux that is mediated by NSCC (Demidchik et al 2002, 2003) and that is largely independent of membrane voltage. Thus a plant's ability to either prevent hydroxyl radicals formation in the first instance, or efficiently scavenge it (by means of non-enzymatic antioxidants) may be critical to plant tolerance under combined WL/NaCl stress conditions. This aspect should be given a highest priority in future studies.

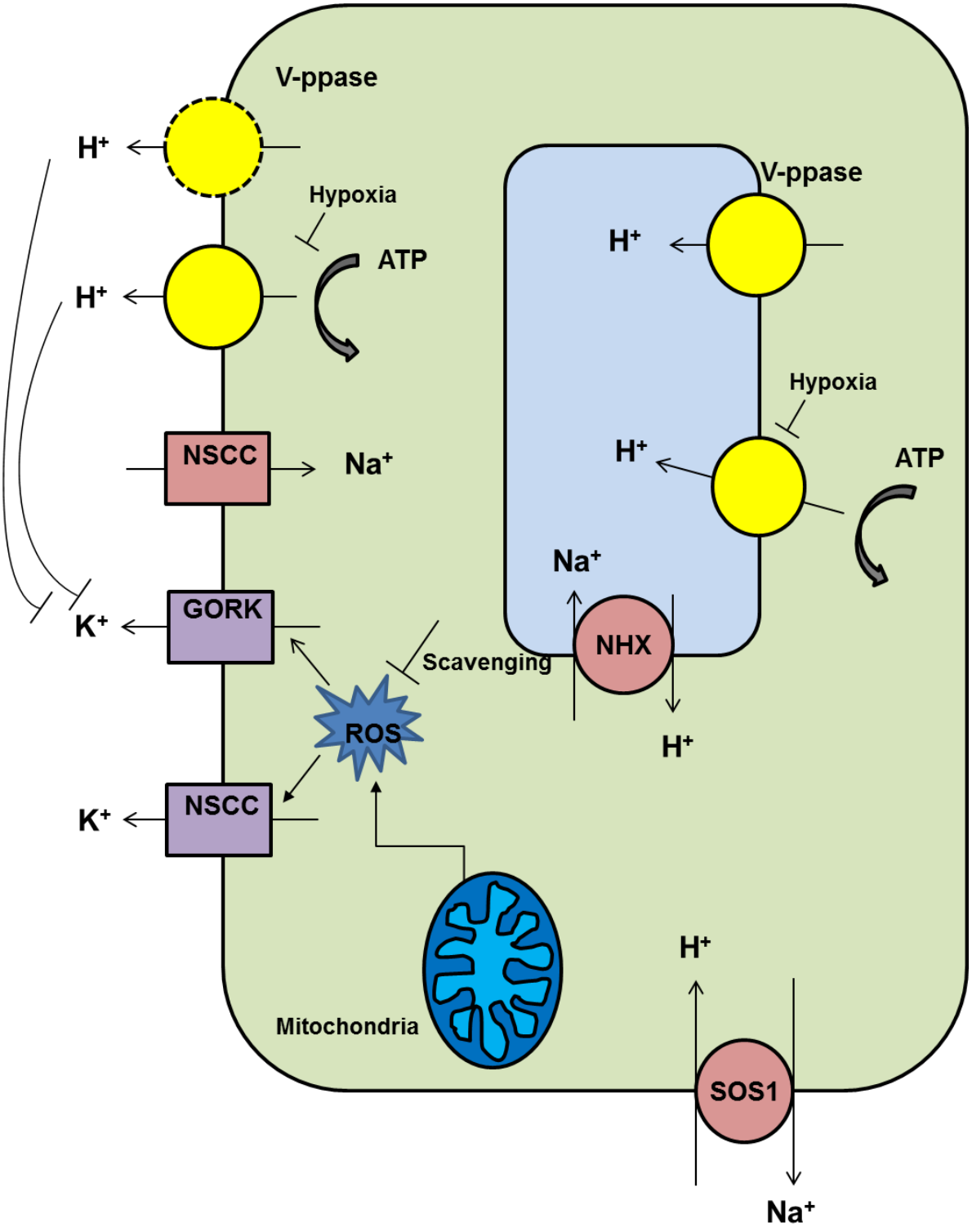

Figure 7.1. A schematic representation of major transporters involved in plant responses to combined salinity and waterlogging stress at the plasma membrane and tonoplast membranes of plant roots.

References

Uncategorized References

Ache P, Becker D, Ivashikina N, Dietrich P, Roelfsema MRG, Hedrich R (2000) GORK, a delayed outward rectifier expressed in guard cells of Arabidopsis thaliana, is a K+-selective, K+-sensing ion channel. Febs Letters 486 (2):93-98

Adem GD, Bose J, Zhou M, Shabala S (2015) targeting Vacuolar Sodium Sequestration in plant Breeding for Salinity tolerance. Managing Salt Tolerance in Plants: Molecular and Genomic Perspectives:35

Alscher RG, Erturk N, Heath LS (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. Journal of experimental botany 53 (372):1331-1341

Amtmann A, Beilby MJ (2010) The role of ion channels in plant salt tolerance. In: Ion Channels and Plant Stress Responses. Springer, pp 23-46

Amtmann A, Fischer M, Marsh EL, Stefanovic A, Sanders D, Schachtman DP (2001) The wheat cDNA LCT1 generates hypersensitivity to sodium in a salt-sensitive yeast strain. Plant Physiology 126 (3):1061-1071

Anil VS, Krishnamurthy P, Kuruvilla S, Sucharitha K, Thomas G, Mathew M (2005) Regulation of the uptake and distribution of Na+ in shoots of rice (Oryza sativa) variety Pokkali: role of Ca2+ in salt tolerance response. Physiologia Plantarum 124 (4):451-464

Anschütz U, Becker D, Shabala S (2014) Going beyond nutrition: regulation of potassium homoeostasis as a common denominator of plant adaptive responses to environment. Journal of Plant Physiology 171 (9):670-687

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373-399

Arikado H, Adachi Y (1955) Anatomical and ecological responses of barley and some forage crops to the flooding treatment. Bull Fac Agric Mie Univ 11:1-29

Armstrong J, Armstrong W (1999) Phragmites die-back: Toxic effects of propionic, butyric and caproic acids in relation to pH. New Phytologist 142 (2):201-217

Armstrong W (1979) Aeration in higher plants.

Assessment ADS (2000) Extent, impacts, processes, monitoring and management options. National Land and Water Resources Audit, Canberra

Bailey-Serres J, Chang R (2005) Sensing and signalling in response to oxygen deprivation in plants and other organisms. Annals of Botany 96 (4):507-518

Bailey-Serres J, Voesenek L (2008) Flooding stress: acclimations and genetic diversity. Annu Rev Plant Biol 59:313-339

Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu Rev Plant Biol 59:89-113

Balnokin YV, Myasoedov N, Shamsutdinov ZS, Shamsutdinov N (2005) Significance of Na+ and K+ for sustained hydration of organ tissues in ecologically distinct halophytes of the family Chenopodiaceae. Russian Journal of Plant Physiology 52 (6):779-787

Barrett-Lennard E (1986) Effects of waterlogging on the growth and NaCl uptake by vascular plants under saline conditions. Reclamation and Revegetation Research (Netherlands)

Barrett-Lennard E (2003) The interaction between waterlogging and salinity in higher plants: causes, consequences and implications. Plant and Soil 253 (1):35-54

Barrett-Lennard E, Ratingen Pv, Mathie MH (1999) Referees 1998-Crop improvement and protection-The developing pattern of damage in wheat (Triticum aestivum L) due to the combined stresses of salinity and hypoxia: Experiments under controlled. Australian Journal of Agricultural Research 50 (2):129-136

Barrett-Lennard EG, Shabala SN (2013) The waterlogging/salinity interaction in higher plants revisited–focusing on the hypoxia-induced disturbance to K+ homeostasis. Functional Plant Biology 40 (9):872-882

Baxter-Burrell A, Yang Z, Springer PS, Bailey-Serres J (2002) RopGAP4-dependent Rop GTPase rheostat control of Arabidopsis oxygen deprivation tolerance. Science 296 (5575):2026-2028

Beckett P, Armstrong W, Justin S, Armstrong J (1988) On the relative importance of convective and diffusive gas flows in plant aeration. New Phytologist 110 (4):463-468

Bennett SJ, Barrett-Lennard E, Colmer T (2009) Salinity and waterlogging as constraints to saltland pasture production: a review. Agriculture, Ecosystems & Environment 129 (4):349-360

Bhandal IS, Malik C (1988) in the Physiology and Metabolism of Flowering Plants. International Review of Cytology 110:205

Blaha G, Stelzl U, Spahn C, Agrawal RK, Frank J, Nierhaus KH (1999) Preparation of functional ribosomal complexes and effect of buffer conditions on tRNA positions observed by cryoelectron microscopy. Methods in enzymology 317:292-309

Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Annals of botany 91 (2):179-194

Bolhar-Nordenkampf H, Long S, Baker N, Oquist G, Schreiber U, Lechner E (1989) Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. Functional Ecology:497-514

Bose J, Rodrigo-Moreno A, Shabala S (2013) ROS homeostasis in halophytes in the context of salinity stress tolerance. Journal of Experimental Botany:ert430

Bose J, Shabala L, Pottosin I, Zeng F, VELARDE-BUENDÍA A, Massart A, Poschenrieder C, Hariadi Y, Shabala S (2014) Kinetics of xylem loading, membrane potential maintenance, and sensitivity of K+-permeable channels to reactive oxygen species: physiological traits that differentiate salinity tolerance between pea and barley. Plant, cell & environment 37 (3):589-600

Buwalda F, Barrett-Lennard E, Greenway H, Davies B (1988a) Effects of growing wheat in hypoxic nutrient solutions and of subsequent transfer to aerated solutions. II. Concentrations and uptake of nutrients and sodium in shoots and roots. Functional Plant Biology 15 (4):599-612

Buwalda F, Thomson C, Steigner W, Barrett-Lennard E, Gibbs J, Greenway H (1988b) Hypoxia induces membrane depolarization and potassium loss from wheat roots but does not increase their permeability to sorbitol. Journal of Experimental Botany 39 (9):1169-1183

Byrt CS, Platten JD, Spielmeyer W, James RA, Lagudah ES, Dennis ES, Tester M, Munns R (2007) HKT1; 5-like cation transporters linked to Na+ exclusion loci in wheat, Nax2 and Kna1. Plant Physiology 143 (4):1918-1928

Cakirlar H, Bowling D (1981) The effect of salinity on the membrane potential of sunflower roots. Journal of Experimental Botany 32 (3):479-485

Cheeseman JM, Hanson JB (1979) Energy-linked potassium influx as related to cell potential in corn roots. Plant Physiology 64 (5):842-845

Chen Z, Cuin TA, Zhou M, Twomey A, Naidu BP, Shabala S (2007a) Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance. Journal of Experimental Botany 58 (15-16):4245-4255

Chen Z, Newman I, Zhou M, Mendham N, Zhang G, Shabala S (2005) Screening plants for salt tolerance by measuring K+ flux: a case study for barley. Plant, Cell & Environment 28 (10):1230-1246

Chen Z, Pottosin II, Cuin TA, Fuglsang AT, Tester M, Jha D, Zepeda-Jazo I, Zhou M, Palmgren MG, Newman IA (2007b) Root plasma membrane transporters controlling K+/Na+ homeostasis in salt-stressed barley. Plant Physiology 145 (4):1714-1725

Chen Z, Shabala S, Mendham N, Newman I, Zhang G, Zhou M (2008) Combining ability of salinity tolerance on the basis of NaCl-induced K flux from roots of barley. Crop Science 48 (4):1382-1388

Chérel I, Lefoulon C, Boeglin M, Sentenac H (2014) Molecular mechanisms involved in plant adaptation to low K+ availability. Journal of experimental botany 65 (3):833-848

Colmer T (2003) Long-distance transport of gases in plants: a perspective on internal aeration and radial oxygen loss from roots. Plant, Cell & Environment 26 (1):17-36

Colmer T, Munns R, Flowers T (2006) Improving salt tolerance of wheat and barley: future prospects. Animal Production Science 45 (11):1425-1443

Colmer TD, Flowers TJ (2008) Flooding tolerance in halophytes. New Phytologist 179 (4):964-974

Colmer TD, Gibberd MR, Wiengweera A, Tinh TK (1998) The barrier to radial oxygen loss from roots of rice (Oryza sativa L.) is induced by growth in stagnant solution. Journal of Experimental Botany 49 (325):1431-1436

Contardi PJ, Davis RF (1978) Membrane Potential in Phaeoceros laevis Effects of Anoxia, External Ions, Light, and Inhibitors. Plant physiology 61 (2):164-169

Cosentino C, Fischer-Schliebs E, Bertl A, Thiel G, Homann U (2010) Na+/H+ antiporters are differentially regulated in response to NaCl stress in leaves and roots of Mesembryanthemum crystallinum. New Phytologist 186 (3):669-680

Cuin TA, Betts SA, Chalmandrier R, Shabala S (2008) A root's ability to retain K+ correlates with salt tolerance in wheat. Journal of Experimental Botany 59 (10):2697-2706

DaMatta F, Hemantaranjan A (2003) Advances in Plant Physiology. Advances in Plant Physiology 5

Davenport R, James RA, Zakrisson-Plogander A, Tester M, Munns R (2005) Control of sodium transport in durum wheat. Plant Physiology 137 (3):807-818

Davenport RJ, MUÑOZ-MAYOR A, Jha D, Essah PA, Rus A, Tester M (2007) The Na+ transporter AtHKT1; 1 controls retrieval of Na+ from the xylem in Arabidopsis. Plant, Cell & Environment 30 (4):497-507

De Boer A, Volkov V (2003) Logistics of water and salt transport through the plant: structure and functioning of the xylem. Plant, Cell & Environment 26 (1):87-101

Demidchik V (2007) 16 Physiological Roles of Nonselective Cation Channels in the Plasma. Communication in Plants: Neuronal Aspects of Plant Life:235

Demidchik V (2012) Reactive oxygen species and oxidative stress in plants. Plant stress physiology CAB International, Oxfordshire:24-58

Demidchik V (2015) Mechanisms of oxidative stress in plants: from classical chemistry to cell biology. Environmental and Experimental Botany 109:212-228

Demidchik V, Cuin TA, Svistunenko D, Smith SJ, Miller AJ, Shabala S, Sokolik A, Yurin V (2010) Arabidopsis root K+-efflux conductance activated by hydroxyl radicals: single-channel properties, genetic basis and involvement in stress-induced cell death. Journal of Cell Science 123 (9):1468-1479

Demidchik V, Maathuis FJ (2007) Physiological roles of nonselective cation channels in plants: from salt stress to signalling and development. New Phytologist 175 (3):387-404

Demidchik V, Shabala SN, Coutts KB, Tester MA, Davies JM (2003) Free oxygen radicals regulate plasma membrane Ca2+-and K+-permeable channels in plant root cells. Journal of Cell Science 116 (1):81-88

Demidchik V, Tester M (2002) Sodium fluxes through nonselective cation channels in the plasma membrane of protoplasts from Arabidopsis roots. Plant physiology 128 (2):379-387

Donnan WW, Houston CE (1967) Drainage related to irrigation management. Irrigation of Agricultural Lands (irrigationofagr):974-987

Drew M (1983) Plant injury and adaptation to oxygen deficiency in the root environment: a review. Plant and soil 75 (2):179-199

Drew M, Lynch JM (1980) Soil anaerobiosis, microorganisms, and root function. Annual Review of Phytopathology 18 (1):37-66

Drew MC (1997) Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. Annual review of plant biology 48 (1):223-250

Drew MC, Läuchli A (1985) Oxygen-dependent exclusion of sodium ions from shoots by roots of Zea mays (cv Pioneer 3906) in relation to salinity damage. Plant Physiology 79 (1):171-176

Dreyer I, Uozumi N (2011) Potassium channels in plant cells. FEBS Journal 278 (22):4293-4303

Dufey I, Hakizimana P, Draye X, Lutts S, Bertin P (2009) QTL mapping for biomass and physiological parameters linked to resistance mechanisms to ferrous iron toxicity in rice. Euphytica 167 (2):143-160

Dulloo E, Hall S, FAO EM, Nasi R (2013) Draft 25 March 2013.

Evans DE (2004) Aerenchyma formation. New Phytologist 161 (1):35-49

Fagerstedt KV, Kukkola EM, Koistinen VV, Takahashi J, Marjamaa K (2010) Cell wall lignin is polymerised by class III secretable plant peroxidases in Norway spruce. Journal of Integrative Plant Biology 52 (2):186-194

Faiyue B, Vijayalakshmi C, Nawaz S, Nagato Y, Taketa S, Ichii M, AL-AZZAWI MJ, Flowers TJ (2010) Studies on sodium bypass flow in lateral rootless mutants lrt1 and lrt2, and crown rootless mutant crl1 of rice (Oryza sativa L.). Plant, cell & environment 33 (5):687-701

FAO (2011) The State of the World's Land and Water Resources for Food and Agriculture, Managing Systems at Risk. The Food and Agriculture Organization of United Nations and Earthscan.

Felle HH (2005) pH regulation in anoxic plants. Annals of Botany 96 (4):519-532

Flowers T, Troke P, Yeo A (1977) The mechanism of salt tolerance in halophytes. Annual review of plant physiology 28 (1):89-121

Flowers T, Yeo A (1992) Transport from Root to Shoot. In: Solute Transport in Plants. Springer, pp 120-142

Flowers TJ, Colmer TD (2008) Salinity tolerance in halophytes*. New Phytologist 179 (4):945-963

Flowers TJ, Galal HK, Bromham L (2010) Evolution of halophytes: multiple origins of salt tolerance in land plants. Functional Plant Biology 37 (7):604-612

Fukuda A, Yazaki Y, Ishikawa T, Koike S, Tanaka Y (1998) Na+/H+ antiporter in tonoplast vesicles from rice roots. Plant and Cell Physiology 39 (2):196-201

Gambrell R, Patrick Jr W (1978) Chemical and microbiological properties of anaerobic soils and sediments. Plant life in anaerobic environments:375-423

Gambrell R, Wiesepape J, Patrick Jr W, Duff M (1991) The effects of pH, redox, and salinity on metal release from a contaminated sediment. Water, Air, and Soil Pollution 57 (1):359-367

Garthwaite AJ, Armstrong W, Colmer TD (2008) Assessment of O2 diffusivity across the barrier to radial O2 loss in adventitious roots of Hordeum marinum. New Phytologist 179 (2):405-416

Garthwaite AJ, von Bothmer R, Colmer TD (2003) Diversity in root aeration traits associated with waterlogging tolerance in the genus Hordeum. Functional Plant Biology 30 (8):875-889

Ghassemi F, Jakeman AJ, Nix HA (1995) Salinisation of land and water resources: human causes, extent, management and case studies. CAB international,

Gibbs J, Greenway H (2003) Review: mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism. Functional Plant Biology 30 (3):353-353

Gitelson AA, Gritz Y, Merzlyak MN (2003) Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves. Journal of plant physiology 160 (3):271-282

Gong H, Randall D, Flowers T (2006) Silicon deposition in the root reduces sodium uptake in rice (Oryza sativa L.) seedlings by reducing bypass flow. Plant, Cell & Environment 29 (10):1970-1979

Greenway H, Osmond C (1972) Salt responses of enzymes from species differing in salt tolerance. Plant Physiology 49 (2):256-259

Haas M (1989) Properties and diversity of (Na-K-C1) cotransporters. Annual Review of Physiology 51 (1):443-457

Haboudane D, Miller JR, Tremblay N, Zarco-Tejada PJ, Dextraze L (2002) Integrated narrow-band vegetation indices for prediction of crop chlorophyll content for application to precision agriculture. Remote sensing of environment 81 (2):416-426

Hald PM (1947) The flame photometer for the measurement of sodium and potassium in biological materials. Journal of Biological Chemistry 167:499-510

Halliwell B (1987) Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. Chemistry and Physics of lipids 44 (2):327-340

Hasegawa PM, Bressan RA, Zhu J-K, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annual review of plant biology 51 (1):463-499

Horie T, Hauser F, Schroeder JI (2009) HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants. Trends in plant science 14 (12):660-668

Horie T, Motoda J, Kubo M, Yang H, Yoda K, Horie R, Chan WY, Leung HY, Hattori K, Konomi M (2005) Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na+ unloading from xylem vessels to xylem parenchyma cells. The Plant Journal 44 (6):928-938

Horie T, Yoshida K, Nakayama H, Yamada K, Oiki S, Shinmyo A (2001) Two types of HKT transporters with different properties of Na+ and K+ transport in Oryza sativa. The Plant Journal 27 (2):129-138

Huang X, Shabala S, Shabala L, Rengel Z, Wu X, Zhang G, Zhou M (2015) Linking waterlogging tolerance with Mn2+ toxicity: a case study for barley. Plant Biology 17 (1):26-33

Hughes Jr FM, Cidlowski JA (1999) Potassium is a critical regulator of apoptotic enzymes in vitro and in vivo. Advances in enzyme regulation 39 (1):157-171

Jackson M (2004) The impact of flooding stress on plants and crops. URL Avaialble at: http://www plantstress com/Articles/(waterlogging)[accessed July 2008]

Jackson MB (1985) Ethylene and responses of plants to soil waterlogging and submergence. Annual Review of Plant Physiology 36 (1):145-174

James RA, Davenport RJ, Munns R (2006) Physiological characterization of two genes for Na+ exclusion in durum wheat, Nax1 and Nax2. Plant physiology 142 (4):1537-1547

John C, Limpinuntana V, Greenway H (1977) Interaction of salinity and anaerobiosis in barley and rice. Journal of Experimental Botany 28 (1):133-141

Jung J-Y, Shin R, Schachtman DP (2009) Ethylene mediates response and tolerance to potassium deprivation in Arabidopsis. The Plant Cell 21 (2):607-621
Kato-Noguchi H (2002) Ethanol sensitivity of rice and oat coleoptiles. Physiologia plantarum 115 (1):119-124
Kautsky H, Hirsch A (1931) Neue versuche zur kohlensäureassimilation. Naturwissenschaften 19 (48):964-964
Kaye Y, Golani Y, Singer Y, Leshem Y, Cohen G, Ercetin M, Gillaspy G, Levine A (2011) Inositol polyphosphate 5-phosphatase7 regulates the production of reactive oxygen species and salt tolerance in Arabidopsis. Plant physiology 157 (1):229-241
Kirk G, Ahmad A, Nye P (1990) Coupled diffusion and oxidation of ferrous iron in soils. II. A model of the diffusion and reaction of O2, Fe2+, H+ and HCO3− in soils and a sensitivity analysis of the model. Journal of soil science 41 (3):411-431
Kordan H (1974) Patterns of shoot and root growth in rice seedlings germinating under water. Journal of Applied Ecology:685-690
Krause GH, Weis E (1984) Chlorophyll fluorescence as a tool in plant physiology. Photosynthesis research 5 (2):139-157
Lacombe B, Pilot G, Gaymard F, Sentenac H, Thibaud J-B (2000) pH control of the plant outwardly-rectifying potassium channel SKOR. FEBS letters 466 (2):351-354
Laohavisit A, Davies JM (2007) The gas that opens gates: calcium channel activation by ethylene. New Phytologist 174 (3):470-473
Laurie S, Feeney KA, Maathuis FJ, Heard PJ, Brown SJ, Leigh RA (2002) A role for HKT1 in sodium uptake by wheat roots. The Plant Journal 32 (2):139-149
Lee R (2011) The outlook for population growth. Science 333 (6042):569-573
Liang Y (1999) Effects of silicon on enzyme activity and sodium, potassium and calcium concentration in barley under salt stress. Plant and Soil 209 (2):217-224
Lichtenthaler HK, Miehe JA (1997) Fluorescence imaging as a diagnostic tool for plant stress. Trends in Plant Science 2 (8):316-320
Lynch J (1977) Phytotoxicity of acetic acid produced in the anaerobic decomposition of wheat straw. Journal of Applied Bacteriology 42 (1):81-87
Ma L, Zhang H, Sun L, Jiao Y, Zhang G, Miao C, Hao F (2011) NADPH oxidase AtrbohD and AtrbohF function in ROS-dependent regulation of Na+/K+ homeostasis in Arabidopsis under salt stress. Journal of Experimental Botany:err280
Maathuis FJ, Amtmann A (1999) K+ nutrition and Na+ toxicity: the basis of cellular K+/Na+ ratios. Annals of Botany 84 (2):123-133
Madigan MT, Martinko JM, Parker J (2003) Microbial growth. Brock biology of microorganisms:137-166
Maggio A, Hasegawa PM, Bressan RA, Consiglio MF, Joly RJ (2001) Review: Unravelling the functional relationship between root anatomy and stress tolerance. Functional Plant Biology 28 (10):999-1004
Malik AI, English JP, Colmer TD (2009) Tolerance of Hordeum marinum accessions to O2 deficiency, salinity and these stresses combined. Annals of Botany 103 (2):237-248
Mancuso S, Shabala S (2010) Waterlogging signalling and tolerance in plants. Springer,
Marschner H (1995a) Adaptation of plants to adverse chemical soil conditions. Mineral nutrition of higher plants 596:680
Marschner H (1995b) Mineral Nutrition of Higher Plants Academic Press London 889.
Marschner H (2011) Marschner's mineral nutrition of higher plants. Academic press,
Mäser P, Eckelman B, Vaidyanathan R, Horie T, Fairbairn DJ, Kubo M, Yamagami M, Yamaguchi K, Nishimura M, Uozumi N (2002) Altered shoot/root Na+ distribution and bifurcating salt sensitivity in Arabidopsis by genetic disruption of the Na+ transporter AtHKT1. FEBS letters 531 (2):157-161

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. Journal of experimental botany 51 (345):659-668

McDonald M, Galwey N, Colmer T (2001) Waterlogging tolerance in the tribe Triticeae: the adventitious roots of Critesion marinum have a relatively high porosity and a barrier to radial oxygen loss. Plant, Cell & Environment 24 (6):585-596

Meng Y, Ren P, Ma X, Li B, Bao Q, Zhang H, Wang J, Bai J, Wang H (2016) GGE Biplot-Based Evaluation of Yield Performance of Barley Genotypes across Different Environments in China. Journal of Agricultural Science and Technology 18 (2):533-543

Miernyk JA (1990) Glycolysis, the oxidative pentose phosphate pathway and anaerobic respiration. Plant physiology, biochemistry and molecular biology:77-100

Miller G, Suzuki N, CIFTCI-YILMAZ S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant, cell & environment 33 (4):453-467

Møller IS, Gilliham M, Jha D, Mayo GM, Roy SJ, Coates JC, Haseloff J, Tester M (2009) Shoot Na+ exclusion and increased salinity tolerance engineered by cell type–specific alteration of Na+ transport in Arabidopsis. The Plant Cell 21 (7):2163-2178

Møller IS, Tester M (2007) Salinity tolerance of Arabidopsis: a good model for cereals? Trends in plant science 12 (12):534-540

Morard P, Silvestre J (1996) Plant injury due to oxygen deficiency in the root environment of soilless culture: A review. Plant and Soil 184 (2):243-254

Mujer CV, Rumpho ME, Lin J-J, Kennedy RA (1993) Constitutive and inducible aerobic and anaerobic stress proteins in the Echinochloa complex and rice. Plant Physiology 101 (1):217-226

Munns R (2002) Comparative physiology of salt and water stress. Plant, cell & environment 25 (2):239-250

Munns R (2005) Genes and salt tolerance: bringing them together. New phytologist 167 (3):645-663

Munns R, Schachtman D, Condon A (1995) The significance of a two-phase growth response to salinity in wheat and barley. Functional Plant Biology 22 (4):561-569

Munns R, Termaat A (1986) Whole-plant responses to salinity. Functional Plant Biology 13 (1):143-160

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651-681

Naidoo G, Mundree S (1993) Relationship between morphological and physiological responses to waterlogging and salinity in Sporobolus virginicus (L.) Kunth. Oecologia 93 (3):360-366

Nass R, Cunningham KW, Rao R (1997) Intracellular Sequestration of Sodium by a Novel Na+/H+ Exchanger in Yeast Is Enhanced by Mutations in the Plasma Membrane H+-ATPase insights into mechanisms of sodium tolerance. Journal of Biological Chemistry 272 (42):26145-26152

Nass R, Rao R (1998) Novel localization of a Na+/H+ exchanger in a late endosomal compartment of yeast Implications for vacuole biogenesis. Journal of Biological Chemistry 273 (33):21054-21060

Newman I (2001) Ion transport in roots: measurement of fluxes using ion-selective microelectrodes to characterize transporter function. Plant, cell & environment 24 (1):1-14

Nishiuchi S, Yamauchi T, Takahashi H, Kotula L, Nakazono M (2012) Mechanisms for coping with submergence and waterlogging in rice. Rice 5 (1):2

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annual review of plant biology 49 (1):249-279
Oh D-H, Lee SY, Bressan RA, Yun D-J, Bohnert HJ (2010) Intracellular consequences of SOS1 deficiency during salt stress. Journal of Experimental Botany 61 (4):1205-1213
Ozgur R, Uzilday B, Sekmen AH, Turkan I (2013) Reactive oxygen species regulation and antioxidant defence in halophytes. Functional Plant Biology 40 (9):832-847
Palmgren MG, Harper JF (1999) Pumping with plant P-type ATPases. Journal of Experimental Botany 50 (Special Issue):883-893
Parlanti S, Kudahettige N, Lombardi L, Mensuali-Sodi A, Alpi A, Perata P, Pucciariello C (2011) Distinct mechanisms for aerenchyma formation in leaf sheaths of rice genotypes displaying a quiescence or escape strategy for flooding tolerance. Annals of botany 107 (8):1335-1343
Perata P, Alpi A (1991) Ethanol metabolism in suspension cultured carrot cells. Physiologia Plantarum 82 (1):103-108
Perata P, Alpi A (1993) Plant responses to anaerobiosis. Plant Science 93 (1):1-17
Petruzzelli L, Sturaro M, Mainieri D, LEUBNER-METZGER G (2003) Calcium requirement for ethylene-dependent responses involving 1-aminocyclopropane-1-carboxylic acid oxidase in radicle tissues of germinated pea seeds*. Plant, Cell & Environment 26 (5):661-671
Pilot G, Gaymard F, Mouline K, Chérel I, Sentenac H (2003) Regulated expression of Arabidopsis Shaker K+ channel genes involved in K+ uptake and distribution in the plant. Plant molecular biology 51 (5):773-787
Plekhanova I (2007) Transformation of Fe, Mn, Co, and Ni compounds in humic podzols at different moisture. Biology Bulletin 34 (1):67-75
Plett D, Safwat G, Gilliham M, Møller IS, Roy S, Shirley N, Jacobs A, Johnson A, Tester M (2010) Improved salinity tolerance of rice through cell type-specific expression of AtHKT1; 1. PLoS One 5 (9):e12571
Qadir M, Tubeileh A, Akhtar J, Larbi A, Minhas P, Khan M (2008) Productivity enhancement of salt-affected environments through crop diversification. Land degradation & development 19 (4):429-453
Qiu Q-S, Barkla BJ, Vera-Estrella R, Zhu J-K, Schumaker KS (2003) Na+/H+ exchange activity in the plasma membrane of Arabidopsis. Plant Physiology 132 (2):1041-1052
Qureshi R, Barrett-Lennard E (1998) Saline agriculture for irrigated land in Pakistan: a handbook. Australian Centre for International Agricultural Research (ACIAR),
Ren Z-H, Gao J-P, Li L-G, Cai X-L, Huang W, Chao D-Y, Zhu M-Z, Wang Z-Y, Luan S, Lin H-X (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nature genetics 37 (10):1141-1146
Rengasamy P (2002) Transient salinity and subsoil constraints to dryland farming in Australian sodic soils: an overview. Animal Production Science 42 (3):351-361
Rengasamy P (2006) World salinization with emphasis on Australia. Journal of Experimental Botany 57 (5):1017-1023
Riadh K, Wided M, Hans-Werner K, Chedly A (2010) Responses of halophytes to environmental stresses with special emphasis to salinity. Advances in Botanical Research 53:117-145
Richards LA (1954) Diagnosis and improvement of saline and alkali soils. Soil Science 78 (2):154
Robertson G (1996) Saline land in Australia-its extent and predicted trends. Australian Journal of Soil and Water Conservation 9:4-7

Rodríguez-Rosales MP, Gálvez FJ, Huertas R, Aranda MN, Baghour M, Cagnac O, Venema K (2009) Plant NHX cation/proton antiporters. Plant signaling & behavior 4 (4):265-276

Ruan C-J, da Silva JAT, Mopper S, Qin P, Lutts S (2010) Halophyte improvement for a salinized world. Critical Reviews in Plant Sciences 29 (6):329-359

Sachs MM, Freeling M, Okimoto R (1980) The anaerobic proteins of maize. Cell 20 (3):761-767

Sairam R, Kumutha D, Ezhilmathi K (2009) Waterlogging tolerance: nonsymbiotic haemoglobin-nitric oxide homeostasis and antioxidants. Current Science 96 (5):674-682

Sairam RK, Dharmar K, Lekshmy S, Chinnusamy V (2011) Expression of antioxidant defense genes in mung bean (Vigna radiata L.) roots under water-logging is associated with hypoxia tolerance. Acta Physiologiae Plantarum 33 (3):735-744

Saqib M, Akhtar J, Qureshi R (2005) Na+ exclusion and salt resistance of wheat (Triticumaestivum) in saline-waterlogged conditions are improved by the development of adventitious nodal roots and cortical root aerenchyma. Plant Science 169 (1):125-130

Scandalios JG (1993) Oxygen stress and superoxide dismutases. Plant physiology 101 (1):7

Setter T, Waters I (2003) Review of prospects for germplasm improvement for waterlogging tolerance in wheat, barley and oats. Plant and Soil 253 (1):1-34

Shabala S (2003) Regulation of potassium transport in leaves: from molecular to tissue level. Annals of Botany 92 (5):627-634

Shabala S (2011) Physiological and cellular aspects of phytotoxicity tolerance in plants: the role of membrane transporters and implications for crop breeding for waterlogging tolerance. New Phytologist 190 (2):289-298

Shabala S (2012) Plant stress physiology. Cabi,

Shabala S (2013) Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. Annals of Botany:mct205

Shabala S, Babourina O, Newman I (2000) Ion-specific mechanisms of osmoregulation in bean mesophyll cells. Journal of Experimental Botany 51 (348):1243-1253

Shabala S, Bose J, Fuglsang AT, Pottosin I (2015) On a quest for stress tolerance genes: membrane transporters in sensing and adapting to hostile soils. Journal of experimental botany:erv465

Shabala S, Cuin TA (2008) Potassium transport and plant salt tolerance. Physiologia Plantarum 133 (4):651-669

Shabala S, Cuin TA, Prismall L, Nemchinov LG (2007) Expression of animal CED-9 anti-apoptotic gene in tobacco modifies plasma membrane ion fluxes in response to salinity and oxidative stress. Planta 227 (1):189-197

Shabala S, Demidchik V, Shabala L, Cuin TA, Smith SJ, Miller AJ, Davies JM, Newman IA (2006) Extracellular Ca2+ ameliorates NaCl-induced K+ loss from Arabidopsis root and leaf cells by controlling plasma membrane K+-permeable channels. Plant Physiology 141 (4):1653-1665

Shabala S, Hariadi Y, Jacobsen S-E (2013) Genotypic difference in salinity tolerance in quinoa is determined by differential control of xylem Na+ loading and stomatal density. Journal of Plant Physiology 170 (10):906-914

Shabala S, Mackay A (2011) Ion transport in halophytes. Advances in botanical research 57:151-187

Shabala S, Shabala L (2011) Ion transport and osmotic adjustment in plants and bacteria. Biomolecular concepts 2 (5):407-419

Shabala S, Shabala S, Cuin TA, Pang J, Percey W, Chen Z, Conn S, Eing C, Wegner LH (2010) Xylem ionic relations and salinity tolerance in barley. The Plant Journal 61 (5):839-853

Shi H, Ishitani M, Kim C, Zhu J-K (2000) The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. Proceedings of the national academy of sciences 97 (12):6896-6901

Shi H, Quintero FJ, Pardo JM, Zhu J-K (2002) The putative plasma membrane Na+/H+ antiporter SOS1 controls long-distance Na+ transport in plants. The Plant Cell 14 (2):465-477

Smethurst CF, Garnett T, Shabala S (2005) Nutritional and chlorophyll fluorescence responses of lucerne (Medicago sativa) to waterlogging and subsequent recovery. Plant and Soil 270 (1):31-45

Smethurst CF, Rix K, Garnett T, Auricht G, Bayart A, Lane P, Wilson SJ, Shabala S (2008) Multiple traits associated with salt tolerance in lucerne: revealing the underlying cellular mechanisms. Functional Plant Biology 35 (7):640-650

Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt K, Tignor M, Miller H (2007) Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change, 2007. Cambridge University Press, Cambridge,

Steffens B, Geske T, Sauter M (2011) Aerenchyma formation in the rice stem and its promotion by H2O2. New Phytologist 190 (2):369-378

Steffens B, Kovalev A, Gorb SN, Sauter M (2012) Emerging roots alter epidermal cell fate through mechanical and reactive oxygen species signaling. The Plant Cell 24 (8):3296-3306

Steffens B, Sauter M (2009) Epidermal cell death in rice is confined to cells with a distinct molecular identity and is mediated by ethylene and H2O2 through an autoamplified signal pathway. The Plant Cell 21 (1):184-196

Striker GG (2012) Flooding stress on plants: anatomical, morphological and physiological responses. INTECH Open Access Publisher,

Su Y, Luo W, Lin W, Ma L, Kabir MH (2015) Model of Cation Transportation Mediated by High-Affinity Potassium Transporters (HKTs) in Higher Plants. Biol Proced Online 17 (1)

Taiz L (2013) Agriculture, plant physiology, and human population growth: past, present, and future. Theoretical and Experimental Plant Physiology 25 (3):167-181

Teakle N, Flowers T, Real D, Colmer T (2007) Lotus tenuis tolerates the interactive effects of salinity and waterlogging by 'excluding'Na+ and Cl− from the xylem. Journal of Experimental Botany 58 (8):2169-2180

Teakle N, Real D, Colmer T (2006) Growth and ion relations in response to combined salinity and waterlogging in the perennial forage legumes Lotus corniculatus and Lotus tenuis. Plant and Soil 289 (1-2):369-383

Teakle NL, Amtmann A, Real D, Colmer TD (2010) Lotus tenuis tolerates combined salinity and waterlogging: maintaining O2 transport to roots and expression of an NHX1-like gene contribute to regulation of Na+ transport. Physiologia plantarum 139 (4):358-374

Tester M, Davenport R (2003) Na+ tolerance and Na+ transport in higher plants. Annals of botany 91 (5):503-527

Triantaphylidès C, Krischke M, Hoeberichts FA, Ksas B, Gresser G, Havaux M, Van Breusegem F, Mueller MJ (2008) Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants. Plant physiology 148 (2):960-968

Trought M, Drew M (1980) The development of waterlogging damage in young wheat plants in anaerobic solution cultures. Journal of Experimental Botany 31 (6):1573-1585

Turkan I, Demiral T, Sekmen AH (2013) The regulation of antioxidant enzymes in two Plantago species differing in salinity tolerance under combination of waterlogging and salinity. Functional Plant Biology 40 (5):484-493

U.N. (2011). United Nations, United Nations

Véry A-A, Sentenac H (2002) Cation channels in the Arabidopsis plasma membrane. Trends in plant science 7 (4):168-175

Visser E, Colmer T, Blom C, Voesenek L (2000) Changes in growth, porosity, and radial oxygen loss from adventitious roots of selected mono-and dicotyledonous wetland species with contrasting types of aerenchyma. Plant Cell and Environment 23 (11):1237-1245

Visser EJ, Voesenek LA (2005) Acclimation to soil flooding—sensing and signal-transduction. In: Root Physiology: from Gene to Function. Springer, pp 197-214

Voesenek L, Armstrong W, Bögemann G, Colmer T, McDonald M (1999) A lack of aerenchyma and high rates of radial oxygen loss from the root base contribute to the waterlogging intolerance of Brassica napus. Functional Plant Biology 26 (1):87-93

Wang Y, Wu P, Wu Y, Yan X (2002) Molecular marker analysis of manganese toxicity tolerance in rice under greenhouse conditions. Plant and Soil 238 (2):227-233

WBCSD (2014) Water, food and energy Nexus Challenges. World Business Council for Sustainable Development.

Wegner LH (2010) Oxygen transport in waterlogged plants. In: Waterlogging Signalling and Tolerance in Plants. Springer, pp 3-22

Wegner LH, De Boer AH (1997) Properties of two outward-rectifying channels in root xylem parenchyma cells suggest a role in K+ homeostasis and long-distance signaling. Plant Physiology 115 (4):1707-1719

Wegner LH, Raschke K (1994) Ion channels in the xylem parenchyma of barley roots (A procedure to isolate protoplasts from this tissue and a patch-clamp exploration of salt passageways into xylem vessels. Plant Physiology 105 (3):799-813

Wegner LH, Stefano G, Shabala L, Rossi M, Mancuso S, Shabala S (2011) Sequential depolarization of root cortical and stelar cells induced by an acute salt shock–implications for Na+ and K+ transport into xylem vessels. Plant, cell & environment 34 (5):859-869

Wu H, Zhu M, Shabala L, Zhou M, Shabala S (2014) K+ retention in leaf mesophyll, an overlooked component of salinity tolerance mechanism: a case study for barley. Journal of integrative plant biology

Wu H, Zhu M, Shabala L, Zhou M, Shabala S (2015) K+ retention in leaf mesophyll, an overlooked component of salinity tolerance mechanism: a case study for barley. Journal of integrative plant biology 57 (2):171-185

Yamauchi T, Shimamura S, Nakazono M, Mochizuki T (2013) Aerenchyma formation in crop species: a review. Field Crops Research 152:8-16

Zeng F, Shabala L, Zhou M, Zhang G, Shabala S (2013) Barley responses to combined waterlogging and salinity stress: separating effects of oxygen deprivation and elemental toxicity. Frontiers in plant science 4

Zhang B, Puard M, Couchat P (1990) Effect of hypoxia, acidity and nitrate on inorganic nutrition in rice plants. Plant Physiology and Biochemistry 28 (5):655-661

Zhang H, Turner N, Poole M, Simpson N (2006) Crop production in the high rainfall zones of southern Australia—potential, constraints and opportunities. Animal Production Science 46 (8):1035-1049

Zhao J, Zhi D, Xue Z, Liu H, Xia G (2007) Enhanced salt tolerance of transgenic progeny of tall fescue (Festuca arundinacea) expressing a vacuolar Na+/H+ antiporter gene from Arabidopsis. Journal of plant physiology 164 (10):1377-1383

Zheng C, Jiang D, Liu F, Dai T, Jing Q, Cao W (2009) Effects of salt and waterlogging stresses and their combination on leaf photosynthesis, chloroplast ATP synthesis, and antioxidant capacity in wheat. Plant Science 176 (4):575-582

Zhu M, Zhou M, Shabala L, Shabala S (2015) Linking osmotic adjustment and stomatal characteristics with salinity stress tolerance in contrasting barley accessions. Functional Plant Biology 42 (3):252-263